THE DESMOPLASTIC RESPONSE:

MECHANISMS OF TUMOUR-INDUCED FIBROGENESIS

COLLEEN FEARNS

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ABSTRACT

The main concern of this thesis is with desmoplasia - a process in which excessive connective tissue is deposited in a neoplasm. This is a common phenomenon in neoplasia but one whose mechanisms are poorly understood.

To study the process I used a human malignant melanoma cell line (UCT-Mel 7) that was established in this laboratory and that, when injected into athymic mice, gave rise to tumours that showed a number of interesting features.

Firstly, the tumour induced a marked desmoplastic response as evidenced by a high content of hydroxyproline in tumour lysates, intense staining for reticulin in sections of the tumour and infiltration of the tumour by host mesenchymal cells.

Secondly, the desmoplasia was associated in UCT-Mel 7-derived tumours with an unusual phasic pattern of growth that was related to the *in vitro* passage number of the melanoma cells. On occasions, murine tumours developed at the site of inoculation of human tumour cells.

I have identified 2 possible mechanisms by which UCT-Mel 7 cells could have induced the desmoplastic response: either the tumour cells could have exerted their effect indirectly, i.e. via macrophages, or they could have stimulated the host's stromal cells directly.

UCT-Mel 7 cells were shown to be chemotactic for mouse macrophages and human foreskin fibroblasts were stimulated, in a dose-dependent manner, to synthesize increased amounts of collagen when co-cultured with mouse peritoneal exudate cells. Stimulation could only be effected by direct cell:cell contact; medium conditioned by macrophages was not effective. The amount of stimulation was not dependent on the state of activation of the peritoneal cells nor on the strain of mouse used.

Tumour cells were also found to act directly. Co-culture of UCT-Mel 7 cells and fibroblasts resulted in increased collagen synthesis by the fibroblasts. Increased synthesis of the protein was reflected in an increase in the amount of collagen mRNA.

UCT-Mel 7 cell stimulated in a dose-dependent manner with an absolute requirement for intimate cell:cell contact with the fibroblasts. DNA synthesis was not required. Dexamethasone, retinoic acid and the tumour promoter, phorbol myristate acetate, had significant primary effects on fibroblast collagen synthesis but did not modify the response to melanoma cells. Indomethacin, however, had a minimal primary effect upon the fibroblasts but significantly augmented the melanoma cell effect.

The nature of the stimulatory cell:cell contact is still uncertain. The gap junction inhibitor, α-glycyrrhetinic acid, did not diminish the melanoma cell effect. Preliminary findings suggested that cell-surface proteoglycans may be important. Removal of the proteoglycans with the

inhibitor of proteoglycan assembly, 4-methylumbelliferyl-β-D-xyloside, abrogated the melanoma cell:fibroblast interaction.

Recombinant basic fibroblast growth factor did not seem to be involved in the desmoplastic response. It was of incidental interest to note that this compound inhibited fibroblast collagen synthesis in a manner that was augmented by the concomitant addition of heparin.

A surprising finding was the production of a potent inhibitor of collagen synthesis by superinduced cells of the mouse macrophage cell line, $P388D_1$. This inhibitor has not been fully characterised.

CONTENTS

ABBREVIATIONS

bFGF	- Basic fibroblast growth factor
cAMP	- Cyclic adenosine 3':5'-monophosphate
DB	- Dulbecco's modified Eagle's medium
DB-10	- DB medium with 10% foetal calf serum
DMSO	- Dimethyl sulphoxide
dpm	- disintegrations per minute
ECM	- Extracellular matrix
EDTA	- Ethylene-diamine-tetra acetate
FCS	- Foetal calf serum
GM-CSF	- Granulocyte-macrophage colony stimulating factor
IFN	- Interferon
IL-1	- Interleukin 1
kb	- Kilobases
kD	- Kilodalton
Kd	- Dissociation constant
LPS	- Lipopolysaccharide
mRNA	- Messenger ribonucleic acid
PGI	- Phosphoglucoisomerase
PMA	- Phorbol myristate acetate
ROI	- Reactive oxygen intermediate
RPMI	- Roswell Park Memorial Institute culture medium 1640
SOD	- Superoxide dismutase
TCA	- Trichloroacetic acid
TGF-β	- Transforming growth factor-β
TNF	- Tumour necrosis factor

LITERATURE REVIEW

Desmoplasia

Solid tumours growing *in vivo* invariably comprise two distinct components. The first of these is made up of the neoplastic cells themselves; the second is the complex supporting stroma that consists of mesenchymal cells and the various macromolecular structural elements that characterize connective tissues (Robbins et al., 1984).

The amount of stroma varies from one tumour to another. In anaplastic carcinomas and sarcomas, it is usually scanty and devoid of recognizable arrangement, whereas in most well differentiated malignant tumours and in benign tumours, the stroma is moderate and ordered. Not infrequently one encounters neoplasms, such as the scirrhous carcinomas, that belong to a third category where the stroma is so abundant and dense that it contributes substantially to the mass of the tumour (Willis, 1967). The development of fibrous stromal tissue within and around a tumour is known as desmoplasia (from the Greek desmos = ligament or band)(Liotta, 1982; Robbins et al., 1984). The extent of this development is referred to as the "desmoplastic response", implying that neoplastic cells will, to a greater or lesser degree, induce the mesenchymal reaction that leads to the deposition of stromal elements.

In recent years there has been a subtle shift in emphasis, away from the narrow view of stromal proliferation as purely a "response" to

tumour cells, to the concept of an interdependence between the parenchyma and the stroma in which the latter may govern such attributes of the former as growth rate, differentiation and invasion. The stromal cells - fibroblasts and endothelial cells - grow in response to mitogenic factors produced by the tumour cells. Tumour cell growth, in its turn is dependent on the rate at which the endothelial cells can form a vascular system (Steel, 1977). This so-called "stromal reaction" provides the support needed for a rapidly growing cell population (Strauli, 1980). As early as 1958, Vasiliev (1958) noted that invasive growth, whether normal or malignant, requires the proliferation of connective tissue. The formation of undifferentiated connective tissue provides a substratum along which invading cells may migrate; it has chemotactic properties; and it provides a favourable environment for invasion. Furthermore the stromal cells underlying tumours exhibit abnormal growth patterns and altered surface properties (Van den Hooff, 1983). There is, thus, a complex regulatory interplay between the tumour and its host. The desmoplastic response becomes prominent when the host overreacts to the presence of the tumour and produces excessive amounts of connective tissue.

The dense fibrous stroma that surrounds the tumour and characterizes the desmoplastic response is due to excessive production of collagen - mainly Types I and III but also Type V (Barsky et al., 1982; Schurch et al., 1981). Overproduction of other extracellular matrix components, e.g. elastin (Martinez-Hernandez et al., 1980) and

proteoglycans (Iozzo, 1984; Iozzo and Muller-Glauser, 1985), may also be a feature.

Ohtani and Sasano (1983) noted changes in the stromal fibroblasts in human colorectal carcinoma samples, that they referred to as "activation", that correlated with the histological assessment of malignancy. These changes included the nuclear enlargement and prominent rough endoplasmic reticulum that are indicative of actively secreting cells. In adenomas with mild atypia the fibroblasts were mildly activated; in adenomas with severe atypia (carcinoma in situ) they were more so and in invasive adenocarcinomas, showing a desmoplastic response, the fibroblasts showed prominent activation.

Generally speaking, the desmoplastic response to carcinomas is related to the degree of invasion (Ohtani and Sasano, 1983; Schurch et al., 1981) and it would seem that penetration of the basal lamina is a requirement for induction of a stromal reaction.

The source of the stromal elements in neoplasms has been a controversial subject. Workers studying desmoplastic melanomas have suggested the tumour cells as the origin of the excess connective tissue (Bryant et al., 1982; From et al., 1983; Moreno et al., 1986; Valensi, 1979), basing their views upon light and electron microscopic findings. The atypical spindle cells contain melanosomes in various stages of maturation and desmosomes between the spindle cells (both features of melanocytes). They also contain abundant, dilated rough endoplastic reticulum (a feature of fibroblasts) thus supporting the

view that the collagenous matrix is produced by dedifferentiated melanoma cells with fibroblastic features and functions.

Others have favoured the host cells (Barsky et al., 1982; Gullino and Grantham, 1963; Iozzo and Muller-Glauser, 1985; Labrecque et al., 1976; Liotta, 1982; Martinez-Hernandez and Catalano, 1980; Naito et al., 1984). It is now generally accepted that the host cells provide the stroma while the tumour governs the amount that is produced (Gullino and Grantham, 1963) either by a direct effect on surrounding mesenchymal cells or, indirectly, by eliciting a chronic inflammatory response (Labrecque et al., 1976). The putative factors responsible for the induction of desmoplasia have not been identified save for the factors that induce angiogenesis - an important component of desmoplasia (Folkman and Klagsbrun, 1987; Strauli, 1980).

A teleological view of desmoplasia would see it as an attempt by the host to "wall off" the tumour both physically and immunologically (Liotta, 1982). Whether, as is implied by this view, the desmoplastic response is, in fact, beneficial to the host is debatable.

In some instances, a desmoplastic response does appear to be associated with a better prognosis (e.g. in nodular sclerosing Hodgkin's disease (Seemayer et al., 1980) and the experimental results obtained by Dvorak (Dvorak et al., 1979) who showed that Line 1 hepatocarcinoma, which induced a fibroblastic response in guinea pigs, regressed 8-13 days after implantation whereas the Line 10 hepatocarcinoma failed to induce a fibrous response and grew unchecked.

Indirect evidence to suggest that collagen may be important in limiting tumour growth and spread is provided by observations that the induction and growth of tumours is accelerated in lathyritic rats (Schweppe et al., 1965) and that inhibition of the desmoplastic response by the administration of proline analogues (Barsky and Gopalakrishna, 1987) or corticoids (Takahashi and Biempica, 1985) favours invasion and metastatic spread. Furthermore, Liotta et al. (1980), Bauer et al. (1977), Biswas and Toole (1987) have shown that rapidly metastasizing tumours contain high levels of collagenase.

Common clinical experience, however, and a number of well documented studies of the relationship between the desmoplastic response and the invasive phenotype tend to support a contrary view. Although desmoplasia is often a feature of benign as well as malignant tumours, it generally true that desmoplastic tumours are associated with a poorer prognosis than their nondesmoplastic counterparts (Cantin et al., 1982; Conley et al.; de Vita et al., 1985). Patient survival time is shortened; metastases are more frequent and it is known that human scirrhous carcinomas tend to invade vigorously (Strauli, 1980). The collagenous stroma does not constitute an effective barrier: it is either degraded or it accompanies the advance of the tumour.

The intensity of a desmoplastic response tends to be related to the tumour rather than the tissue in which it resides, so that secondary deposits of scirrhous carcinomas are usually desmoplastic in other locations e.g. lung, liver, bone marrow and lymph glands (Willis,

1967; Urmacher, 1984). Occasionally, non desmoplastic metastatic deposits are found (Frolow et al., 1975) and both fibrotic and parenchymatous metastases from the same primary tumour may occur within a single individual (Conley et al., 1971).

Tumours that are commonly desmoplastic include infiltrating ductal carcinoma of the breast, colorectal carcinoma, diffuse mesothelioma and carcinomas of the stomach and pancreas (Barsky et al., 1982; Cantin et al., 1982; Dvorak, 1986). In some cases, the excessive stroma can account for more than 90% of the total tumour mass.

Tumours that are rarely desmoplastic include medullary carcinoma of the breast (Dvorak, 1986) and malignant melanoma, although there have been a number of reports in the literature of a rare variant of the malignant melanoma, the desmoplastic malignant melanoma (Bryant et al., 1982; Conley et al., 1971; Frolow et al., 1975; From et al., 1983; Labrecque et al., 1976; Moreno et al., 1986; Urmacher, 1984; Valensi, 1979).

If one assumes that the amount of connective tissue in a tumour reflects the balance between synthesis and degradation, then clearly the intensity of the desmoplastic response might be influenced by factors that contribute to either side of this equation. Neoplastic induction of increased rates of collagen synthesis has been demonstrated for collagen, both <u>in vitro</u> (Naito et al., 1984) and <u>in vivo</u> (Barsky and Gopalakrishna, 1987; Bano et al., 1983), and for proteoglycans, <u>in vitro</u> (Knudson et al., 1984; Merrilees and Findlay,

1985; Biswas and Toole, 1987) and in vivo (Iozzo and Muller-Glauser, 1985; Toole et al., 1979). In some instances, there has been a documented requirement for direct contact between the tumour cells and the fibroblasts (Knudson et al., 1984) whereas in other cases, tumour cells released uncharacterized soluble factors that were capable of stimulating the fibroblasts (Merrilees and Findlay, 1985). Biswas and Toole (1987) have described experiments in which they isolated the membranous fraction from homogenated cells that induce a desmoplastic response and found that these appeared to be active in stimulating proteoglycan synthesis. In most cases, fibroblasts have been identified as the cells responsible for the increased production of the extracellular matrix, although the tumour cells themselves are able to produce some of these components (Merrilees and Findlay, 1985).

Decreased degradation of the matrix might be due either to decreased synthesis of proteases (Bauer et al., 1986) or to increased synthesis of protease inhibitors. Barsky and Gopalakrishna, for example, have shown that desmoplastic breast carcinomas contain high levels of inhibitors of both Type I and Type IV collagenases (Barsky and Gopalakrishna, 1986).

Increased synthesis and inhibition of degradation are not mutually exclusive and probably act in concert. In a case reported by Bauer et al. (1986) of "progressive nodular fibrosis of the skin", the underlying defects were found to involve both increased synthesis of collagen and decreased levels of collagenase.

Desmoplasia has been likened to the process of wound healing (Dvorak et al., 1984a; Dvorak, 1986) in which the initial fibrin-fibronectin gel is replaced by granulation tissue. This is then remodelled to give predominantly collagenous scar tissue. Fibrin is a regular stromal component of tumours and is present in amounts that are determined by increased permeability of the neoplastic vascular bed (Senger et al., 1983); by the procoagulant activity in tumours (Bach et al., 1981; Dvorak et al., 1983; van de Water et al., 1985); and by local fibrinolytic mechanisms (Dvorak et al., 1984b; Dano et al., 1985). It is well known that the provisional fibrin-fibronectin matrix that is seen in early experimental tumours is replaced by stroma which closely resembles granulation tissue and that this then matures into dense, relatively acellular and poorly vascularised connective tissue that is very similar in appearance to scar tissue (Dvorak et al., 1979; Dvorak et al., 1984a; Schurch et al., 1981). Dvorak and his associates have shown, both in guinea pigs (Dvorak et al., 1979; Dvorak et al., 1984a) and in humans (Dvorak et al., 1981), that there is a strong correlation between the amount of fibrin surrounding a tumour and the magnitude of the desmoplastic response that is induced.

The host stromal response to malignant tumours may, therefore, be regarded as an exaggerated but normal quantitative response rather than as a qualitatively abnormal reaction. It is also a relatively specific phenomenon in the sense that it is found only in some tumours and mainly those of epithelial origin. It is not merely a non-specific reaction by the host to injury or to a "foreign body".

Although the phenomenon is common, the factors and mechanisms involved in desmoplasia are poorly understood and few studies have been specifically designed to investigate the phenomenon.

In this thesis I describe the results of a series of experiments that I performed in an attempt to understand the induction of desmoplasia.

I used, as my experimental system, a human melanoma cell line that was tumorigenic in nude mice, consistently giving rise to slowly growing tumours that were heavily infiltrated with murine fibroblasts, macrophages and collagen. These same cells, when co-cultivated with human fibroblasts *in vitro*, reproducibly induced an increased rate of fibroblast collagen synthesis. Before presenting my results I should like to review the biosynthesis of collagen giving emphasis to the way in which it is regulated and to the factors that affect it.

Collagen Synthesis

Collagen is a major component of connective tissue. With its triple helical rod-like structure and its ability to polymerize into extracellular fibres, it gives interstitial substance to tissues and plays a major role in defining organ structure.

Collagen consists of three polypeptide "α" chains. As synthesized, each α chain can be divided into three domains: an N-terminal propeptide; a lengthy, triple-helical domain flanked by short

non-helical telopeptides; and a C-terminal propeptide (Prockop et al., 1979a).

The triple helical domain contains about 1000 amino acid residues with every third residue being glycine. This can be represented by the general molecular formula $(X\text{-}Y\text{-}Gly)_n$, where X and Y represent amino acids other than glycine. About 100 of the X residues are proline and about 100 of the Y resides are hydroxyproline, making collagen unique in the high concentration of these two residues that it contains. Both proline and hydroxyproline are rigid, cyclic amino acids and thus contribute to the stability of the triple helix. Glycine is the smallest amino acid and occupies the restricted space in the centre of the triple helix. The amino acid residues in the helical portion of the collagen molecule are generally basic. (Prockop et al., 1979a)

Collagen is first synthesised as a longer molecule, procollagen, that contains additional propeptides on both the N-terminal and C-terminal ends of the α chains. These account for about a third of the size of the procollagen molecule. The N-terminal propeptide contains 3 domains: a globular amino-terminal domain, a central collagen-like domain, and another short globular domain. The C-terminal propeptide is globular without any collagen-like domain. Both propeptides contain cysteine, which is not found in the helical portion of the peptide chains. The cysteines are involved in intra- and inter-chain disulphide bonds. The propeptides contain relatively high levels of acidic and hydrophobic residues. The N-terminal propeptide acts as a feedback inhibitor of procollagen synthesis (Wiestner et al., 1979).

The C-terminal propeptide has a role in directing chain association. The interchain disulphide bonds formed in the C-terminal propeptide are an absolute requirement for triple helix formation (Kivirikko and Myllyla, 1979; Prockop et al., 1979a).

Collagen is unique in that it undergoes extensive post-translational processing. Firstly, prolyl residues are hydroxylated to 4-hydroxyproline or 3-hydroxyproline (by the enzymes prolyl 4-hydroxylase (EC 1.14.11.2) or prolyl 3-hydroxylase (EC 1.14.11.?), respectively) and lysyl residues are converted to hydroxylysine (by the enzyme lysyl hydroxylase (EC 1.14.11.4)). All three hydroxylases require ferrous ions, molecular oxygen, α-ketoglutarate and ascorbic acid as co-factors.

The residues are hydroxylated only after their incorporation into the collagen peptide. Prolyl 4-hydroxylase and lysyl hydroxylase act only on proline or lysine residues in the Y position of the X-Y-Gly sequence. In contrast, prolyl 3-hydroxylase acts on proline in the X position but only if the Y position is 4-hydroxyproline. Thus 3-hydroxyproline occurs in sequences of 3-Hyp-4-Hyp-Gly. The hydroxylases act only on the unfolded peptide chains. Further hydroxylation is prevented when the peptide chains fold into their triple-helical conformation.

The function of the hydroxy group of the 4-hydroxyproline residues is to stabilize the triple helix at 37°C (Kivirikko and Myllyla, 1984); α chains which are underhydroxylated cannot form a stable helix and are

rapidly degraded intracellularly. The role of the 3-hydroxyproline is unknown. The hydroxy group of the hydroxylysine has at least 2 functions: it acts as a site for the attachment of the glycosyl units and it is essential for the stability of the intermolecular collagen crosslinks formed during fibril formation (Prockop et al., 1979a; Kivirikko and Myllyla, 1984).

The hydroxylated lysine residues are further modified by glycosylation. The sugar residues, galactose and glucose are added sequentially through O-glycosidic linkages. Galactosyltransferase (EC 2.4.1.50) adds galactose to the hydroxylysine resides and glucosyltransferase (EC 2.4.9.66) adds glucose to the galactosyl-hydroxylysine residues. Both enzymes require the presence of a bivalent cation (preferably manganese) and a nonhelical conformation of the peptide chains. Glycosylation ceases when the collagen peptides fold into a triple helix. The function of the glycosyl residue is uncertain; it is possible that they affect fibril morphology.(Prockop et al., 1979a; Kivirikko and Myllyla, 1984)

Two essential requirements must be met for collagen to assume its triple-helical confirmation. In the first place, there is an absolute need for the formation of inter-chain disulphide bonds. Since these are established between carboxy-terminal propeptides, the triple-helix cannot form until most of the chains have been translated. Secondly, helix formation requires that most, if not all, of the proline residues in the Y position of the X-Y-Gly triplet be 4-hydroxylated (Kivirikko and Myllyla, 1984).

The protein is assembled in the rough endoplasmic reticulum and passes through the Golgi apparatus before being secreted. The rate of secretion depends upon the intracellular processing of the protein and particularly upon the folding of the prochains into a triple helix. (Prockop et al., 1979a; Kivirikko et al., 1984).

Once the procollagen has been secreted it is acted upon by two proteases, both of which require a divalent metal such as calcium. A procollagen aminoprotease removes the aminopropeptides and a procollagen carboxyprotease removes the carboxypropeptides. This proteolytic conversion is essential for normal fibril formation. After assembly to form fibrils the collagen molecules are then crosslinked by a series of covalent bonds that give collagen its tensile strength.

To date, 11 structurally and genetically distinct collagens have been identified and characterised. All are composed of 3 α chains and all contain a region which has the general structure of $(X\text{-}Y\text{-}Gly)_n$, but that varies in amino acid and carbohydrate composition. They have recently been divided into 3 groups according to their size and physicochemical properties. (Miller and Gay, 1987)

Group 1 molecules are composed of chains with a molecular mass of 95 kilodaltons or greater and they are characterized by the presence of a lengthy, uninterrupted helical domain. The group 1 molecules, comprising Types I, II, III, V and K, are the major fibril- and fibre-forming species of collagen.

Type I collagen is the major connective tissue protein of skin, bone and tendon. It consists of two identical α1(I) chains and one α2(I) chain.

Type II collagen is found in hyaline cartilage and consists of 3 identical α chains, α1(II).

Type III collagen is a minor constituent of tissues such as lung, skin and arteries. It consists of 3 α1(III) chains.

Type V and Type K are minor components of the total collagen.

The structure and function of different tissues appear to be intimately associated with the relative amounts of Type I and Type III collagen. Rigid tissues e.g. bone and tendon contain only Type I. More pliable tissues e.g. skin and lung contain both Types I and III.

Group 2 molecules also contain chains of molecular mass 95 kilodaltons or greater but, unlike Group 1 collagens, these contain several helical domains separated by sequences of nonhelical conformation. The Group 2 molecules, including Types IV, VI, VII and VIII, do not readily form fibres but tend to aggregate through end-to-end association forming large networks with an open mesh-like structure. Type IV collagen is associated with basement membranes. It consists of two α1(IV) chain and one α2(IV) chain. This collagen differs from the group 1 collagens in that the globular propeptides are not removed during the extracellular processing of the protein. Much interest has

been focussed on Type IV collagen and the possible role it may play in tumour invasion and metastasis.

Finally, group 3 molecules are composed of chains of molecular weight less than 95 kilodaltons. They consist of Types IX and X.

At least 20 collagen genes have been identified. Present data suggest that each gene is present as a single copy within a haploid set of chromosomes. Collagen genes have been located on chromosome 17 (α1(I)), chromosome 7 (α2(I)), chromosome 12 (α1(II)), chromosome 2 (α1(III) and α2(V)) and chromosome 13 (α1(IV) and α2(IV)). Collagen genes are very large - about 40 kilobases - and can contain up to 51 introns. The gene consists of 6 domains: the signal peptide, 2 terminal propeptides, 2 telopeptides and the triple helical domain (Cheah and Grant, 1982; Ramirez et al., 1985). In the Group 1 collagens, the domain coding for the triple helix has been found to contain a number of exons containing 54 base pairs. There are also exons of 45, 99 and 108 base pairs. These exons encode multiples of the X-Y-Gly triplet. This suggests that the fibrillar collagen genes arose from an ancestral 54 base-pair, primordial gene.

Regulation of collagen biosynthesis

The biosynthesis of collagen may, in theory, be regulated at a number of transcriptional and post-transcriptional steps: gene selection, transcription, RNA processing, translation, post-translational

modification, translocation, secretion, extracellular modification and assembly of collagen fibrils. Collagen can be regulated in terms of both the amount of collagen produced and the type of collagen produced.

Collagen genes have been shown to possess promoter sequences upstream to the transcription initiation site and enhancer sequences within the first intron. (Horton et al., 1987; Rossi and de Crombrugghe, 1987). The promoter squences of the mouse α2(I) collagen gene and the α1(III) collagen gene contain binding sites for nuclear factor 1 (Rossi et al., 1988) indicating that their rate of transcription could be regulated by molecules that interact with these 2 regions. The DNA binding proteins, transcription factor Sp1 and a viral core enhancer, have been shown to inhibit transcription of the α1(I) collagen gene (Bornstein et al., 1987). The authors speculate that a sequence within the first intron of the gene could regulate transcription either positively or negatively depending on the concentration of the relevant DNA binding proteins.

Since the collagen synthesis involves a multi-gene system, production of a functional collagen molecule requires the co-ordinated transcription of the α chain genes situated on different chromosomes e.g. α(I) gene on chromsome 17 and α2(I) gene on chromosome 7 to produce Type I collagen. There also needs to be co-ordinated expression of the collagen structural genes and the genes coding for the various enzymes responsible for post-translational modification.(Olsen, 1981).

Various exogenous factors have been shown to influence collagen gene expression (Kivirikko and Myllyla, 1984). Chrondrocytes, which ordinarily synthesize Type II procollagen exclusively, can be induced to switch to producing Type I procollagen by culturing at low cell density; by shifting from suspension to monolayer culture or by prolonged time in culture. Fibronectin, calcium ions and cyclic AMP also have this effect. This type of gene selection has also been shown in fibroblasts and mouse embryonic carcinoma cells. Fibroblasts can synthesize both Types I and III procollagen simultaneously and the relative proportions of each can be varied. Early granulation tissue, for example, has a very low I:III ratio whilst mature scar tissue has a high I:III ratio (Hance and Crystal, 1977). Mouse embryonic carcinoma cells, when treated with retinoic acid or plated at a low density, switch from producing mainly Type IV collagen to producing Type I collagen (Adamson et al., 1979).

Circumstances that influence the rate of collagen synthesis by a direct effect on transcription are illustrated by infection of chicken embryo fibroblasts with Rous sarcoma virus. The decrease in collagen synthesis that ensues is associated with a decrease in procollagen mRNA (Sandmeyer and Bornstein, 1979).

Regulation at the translational level is more complex and appears to be related to qualitative changes in the collagen mRNA. Chondrocytes, for example, which produce only Type II procollagen, contain mRNA for both Types I and II procollagen. It, thus, appears that the Type I collagen gene is transcribed but is not translated. The mechanisms

involved are uncertain but possible regulatory mechanisms include (i) differential utilization of the mRNA due to structural differences in the mRNAs themselves or to the presence of other regulatory RNA molecules; (ii) differential distribution of mRNA into polysomes (which are translated) or in non-translated ribonucleoprotein complexes (RNPs); or (iii) the stability of the mRNA. There is evidence for all three possibilities.

Processing of the primary collagen mRNA transcript involves some 50 splicing events so that the 28kb transcript of the pro-α2 gene, for example, is reduced to a 5 kb functional mRNA. This provides ample opportunity for differential or regulatory splicing to occur so that differences in processing might lead to altered structural properties of the mRNA which, in turn, might influence translatability of the message. This would explain the observation that the Type I procollagen mRNA produced by chondrocytes migrates with an electrophoretic mobility that is different from that of collagen mRNA from other cell types. (Focht and Adams, 1984).

A well-documented mechanism for translational regulation involves the differential distribution of the mRNA among polysomes and ribonucleoproteins. Actin synthesis in sea urchin embryos is controlled by the mechanisms which regulate this partition (Infante and Heilmann, 1981). Voss and Bornstein (1986) have produced evidence to indicate that collagen mRNAs are not subject to this type of control.

Raghow et al.(1987) have shown that increased intracellular concentrations of procollagen mRNA are not the result of increased transcription of the collagen genes and have suggested that this reflects increased stability of the mRNA molecules.

As has already been mentioned, the N-terminal propeptides exert a negative feedback on collagen synthesis by inhibiting translation of the procollagen mRNAs. (Wiestner et al., 1979). Just how they exert their effect is unknown.

Post-translational regulation (Kivirikko and Myllylla, 1984) is determined by the efficiency of hydroxylation of the prolyl residues and, to a lesser extent, the lysyl residues. The rate of hydroxylation, and the consequent post-translational modifications, is influenced by four factors:

(i) The level of active enzyme present. Tissue prolyl hydroxylase activity fluctuates with the rate of collagen synthesis suggesting that this enzyme is involved in a rate controlling step. In most cells and tissues, a large fraction of the prolyl hydroxylase is found in an inactive form that contains only 1 of the 2 different kinds of subunits present in the active form of the enzyme. Activation of the enzyme could thus regulate the rate of collagen synthesis (Prockop et al., 1979b). The levels of the other post-transcriptional enzymes also vary with the rate of collagen synthesis.

(ii) The availability of co-factors may affect the rate of hydroxylation. Scurvy provides a classical example of this. The deficiency of ascorbate that is associated with this disease leads to underhydroxylation of the procollagen chains which are then degraded intracellularly.

(iii) The rate of procollagen synthesis, by affecting the ratio of enzyme to substrate may influence the rate of hydroxylation. Decreased synthesis gives an increased enzyme:substrate ratio that leads to increased modification of the chains. Such is the case in transformation. Myllyla et al.(1981) have shown that there is an increase in lysyl hydroxylation and an increase in the ratio of glycosylated to nonglycosylated hydroxylysine in 3 human sarcoma cell lines that have reduced rates of procollagen synthesis.

(iv) The rate at which pro-α chains fold into a triple helix influences the degree of modification. If helix formation is delayed increased modification of the pro α chains results (Prockop et al., 1979b; Kivirikko and Myllyla, 1979).

Intracellular degradation of collagen provides another important mechanism for regulating the net rate of accumulation of this protein. This is dramatically illustrated by in vitro experiments that have shown that, during the log phase of growth of subconfluent cell cultures, as much as 30% of the newly-synthesized and under-hydroxylated collagen may be degraded within minutes of synthesis. As growth of the cultures slowed with approaching

confluence, so hydroxylation increased (from 25% in non-confluent to 47% in confluent cultures) and intracellular degradation of newly synthesized collagen decreased to 10% (Berg et al., 1980). This was found for both Type I and Type III collagen. This phenomenon is not unique to collagen since other secretory proteins are also subjected to intracellular degradation e.g. insulin, parathyroid hormones and prolactin (Rennard et al., 1982).

Rennard et al. (1982) have shown that the rate of intracellular Type I collagen degradation is directly related to the intracellular concentration of cAMP. They suggested that cAMP-mediated regulation of intracellular collagen degradation could be a general mechanism whereby a number of external stimuli could influence the rate of collagen accumulation and secretion. Intracellular degradation may play a role both in monitoring defective collagen molecules and in regulating collagen synthesis in response to external stimuli (Baum et al., 1980).

Extracellular matrix: its influence on cell function

Whereas previously the extracellular matrix, comprising collagen, elastin and proteoglycans and glycoproteins was regarded as an inert support medium that served no purpose other than to give shape and tensile strength to tissues or organs, recent experiments have shown that the extracellular matrix plays a far more important role as a modulator of function of the cells that it supports. The composition of the extracellular matrix has profound effects on cell function and

cell-cell interactions, thus influencing cytodifferentiation, mitogenesis, morphogenesis, cell adhesion and migration.

The extracellular matrix has been shown to influence differentiation (Blum et al., 1987; Chen and Bissell, 1987; Reh et al., 1987). In vitro, the extracellular matrix can maintain or restore a differentiated phenotype by increasing the expression of tissue specific genes (as measured by increased mRNA and protein levels) and decreasing the expression of common structural genes (Ben-Ze'ev et al., 1988; Fujita et al., 1987; Spray et al., 1987). The matrix influences gene expression at the level of transcription (increased mRNA produced) and post transcriptionally (e.g. increased mRNA stability or translatability)(Blum et al., 1987; Fujita et al., 1987). This effect is dependent not only on cell-matrix interactions but also on cell-cell interactions. Cells at high density on an unfavourable substrate behave like cells at low density on a favourable substrate (Ben-Ze'ev et al., 1988).

The extracellular matrix influences proliferation in a number of ways. Firstly, it can synergize with growth factors or hormones so that the cells require fewer growth hormones at a lower concentration for optimal growth in vitro (Gatmaitan et al., 1983; Muschel et al., 1986). Secondly, it can influence a cell's response to growth factors (Bano et al., 1985; Madri et al., 1988) e.g. endothelial cells treated with transforming growth factor β (TGF-β) are inhibited when grown on plastic but rapidly form an extensive capillary-like network when grown embedded in collagen (Madri et al., 1988). Thirdly, it can mimic or

mediate the effect of growth factors (Madri et al., 1988; Salomon et al., 1981) e.g. fibronectin can mimic the effect of TGF-β on endothelial cell proliferation. Lastly, the matrix has been shown to sequester growth factors (Folkman et al., 1988; Gordon et al., 1987; Roberts et al., 1988; Vlodavsky et al., 1987). The component of extracellular matrix responsible for binding the growth factors is heparan sulphate. This, for example, could explain the absolute requirement for close contact between bone marrow stromal cells and haematopoietic stem cells during haemopoiesis (Gordon et al., 1987; Roberts et al., 1988).

The extracellular matrix profoundly affects cellular morphology (Ben-Ze'ev et al., 1988; Blum et al., 1987). Epithelial cells are polar, with the basal cell surface adjacent to the basement membrane and the apical surface free of extracellular matrix. This polarity is determined by the matrix (Hall et al., 1982). If the apical surface comes into contact with extracellular matrix, the cells rearrange to exclude the matrix (Chambard et al., 1981; Hall et al., 1982; Montesano et al., 1984). The extracellular matrix is also responsible for determining the shape of the basal cell surface with a co-ordinate change in the organisation of the cytoskeleton (Hay, 1982; Sugrue and Hay, 1981).

Cell adhesion and migration are also influenced by the matrix. Cell-cell adhesion is determined, in part, by cell adhesion molecules (CAMs), specific cell surface glycoproteins that mediate cell-cell adhesion (Cunningham, 1986). Le Douarin (1984) in a review on cell migration in the embryo noted that neural crest cells, which normally

express N-CAM, did not do so during migration. Instead they were surrounded by fibronectin. By altering their surface molecules, cells are able to migrate differentially and to adhere.

Bissell et al. (1982) have described the interaction between cells and their surrounding extracellular matrix as one of "dynamic reciprocity". The response of the cells to the extracellular matrix affects the composition of the new matrix they secrete. This leads to an altered extracellular matrix which, in turn, leads to an altered cellular response. Any molecule capable of altering collagen metabolism could, therefore, have far reaching consequences.

The physiology and pharmacology of control of collagen synthesis

If one takes a less "molecular" and rather more physiological or pharmacological view of the way in which the rate of collagen synthesis is regulated, there appear to be at least four categories of extracellular stimuli that act in this regard. These are provided by:

Lymphokines and monokines

Peptide growth factors

Drugs

Contacts with other cells

There is probably little chemical justification for drawing a distinction between lymphokines and monokines on the one hand and peptide growth factors on the other, since both categories share many molecular characteristics. Lymphokines and monokines, however, are

intuitively associated with immune reactions and the fibrosis that is so frequently seen as part of the host immune response. Peptide growth factors comprise diverse molecules, many of which have only recently been identified and that have not yet been fully characterized in terms of their origin and cellular effect.

Lymphokines and Monokines

Mononuclear cells and closely associated mesenchymal cells are seen in most inflammatory reactions. The mononuclear cells precede the fibroblasts and then exist in close proximity with these cells that infiltrate, divide and produce components of the extracellular matrix. Much attention has been focussed on interactions between fibroblasts and mononuclear cells in attempts to identify the cytokines involved and their mode of action (Freundlich et al., 1986). The situation is complex and many contradictory results have been obtained.

In an *in vitro* system, the results obtained are dependent on a number of variables. The composition of the mononuclear cell population; the agent used for stimulation; the origin and passage number of the fibroblasts used; and the assay system itself (e.g. presence or absence of serum or the time course of the experiment) may all affect the results. Furthermore, earlier experiments used crude or partially purified preparations of the cytokines. With the advent of recombinant cytokines, some of the controversy can now be resolved.

Lymphokines and monokines have been shown to affect several fibroblast functions, namely, fibroblast chemotaxis, proliferation and matrix synthesis. There are both positive and negative modulators of these functions (Wahl, 1984). However, not many of these factors have been well characterised. Of the many cytokines investigated, three are worthy of special mention: interleukin 1, interferon γ and tumour necrosis factor.

Interleukin 1

Interleukin 1 (IL-1) is a 17,5 kD protein produced by macrophages or monocytes in response to stimulation by lipopolysaccharide, immune complexes, phagocytosis and lymphokines (Dinarello and Mier, 1986; Maury, 1986). In a recent review, Oppenheim et al. (1986) noted that IL-1 activity can be produced by virtually all nucleated cell types. It has a wide range of effects, both immunological and non-immunological. It is genetically unrestricted and immunologically non-specific. There are two forms of IL-1, IL-1α and IL-1β, that are products of different genes and have only 26% homology in their amino acid sequence. More than 95% of the IL-1 produced by mononuclear cells is of the IL-1β form. Its effects on tissues of mesenchymal origin may be either reparative e.g. increased synthesis of fibronectin, hyaluronate and collagen or destructive e.g. increased synthesis of collagenase and plasminogen activator (Laato and Heino, 1988). It induces a proliferative response in dermal, synovial, renal, brain and other tissues. This proliferation can lead to fibrosis which results in scarring and organ dysfunction (Dinarello and Mier, 1986). IL-1 is also chemotactic for neutrophils (Sauder et al., 1984), monocytes, B

and T cells (Miossec et al., 1984). Thus it can amplify and potentiate an inflammatory response: the net outcome of which depends on the complex interactions between IL-1 , other cytokines and mesenchymal tissue. The importance of IL-1 in acute inflammatory events is questionable since IL-1 is not stored in cells, but synthesized on demand. Maximal mRNA levels are found 6 h after induction of IL-1 synthesis (Oppenheim et al., 1986): long after acute events have occurred (Billingham, 1987).

IL-1α and IL-1β share a common high affinity (Kd $\pm$ 10^{-10}M) plasma membrane receptor (Kilian et al., 1986). Receptors have been detected on a number of cell types and their presence correlates with the capacity of the cell type tested to respond to IL-1 (Dower et al., 1985). The receptor has recently been cloned and found to belong to the immunoglobulin superfamily along with receptor for other hormones, PDGF and colony stimulating factor (Sims et al., 1988). Natural inhibitors have been isolated and partially characterised from urine (Liao et al., 1985) and neutrophils (Tiku et al., 1986).

The complexity of the interactions between IL-1, other cytokines and mesenchymal tissue is well illustrated by the manner in which IL-1 affects collagen synthesis.

IL-1 can modulate net collagen synthesis in 4 ways:

i) it can increase collagen synthesis by increasing the levels of procollagen mRNA; (Kahari et al., 1987; Postlethwaite et al., 1988)

ii) it can decrease collagen synthesis by increasing the intracellular concentration of prostaglandin E_2; (Laato and Heino, 1988; Postlethwaite et al., 1988)

iii) it can increase the synthesis of collagenase (Postlethwaite et al., 1983) and the tissue inhibitor of metalloproteinases (Postlethwaite et al., 1988) and

iv) it can increase plasminogen activator synthesis which will then activate latent collagenases (Laato and Heino, 1988).

Thus IL-1 can regulate both collagen production and collagen breakdown.

The role of IL-1 as a regulator of collagen synthesis in vivo is brought into question by the absence of the signal sequence (Blobel and Dobberstein, 1975) that is usually required to achieve cellular export of proteins (Auron et al., 1984; March et al., 1985). Some authors have suggested that the IL-1 leaks out when the macrophages are damaged (Gery et al., 1981).

The Interferons

The interferons (IFN) are a family of gene products characterized by their ability to induce resistance to viral infection. They consist of 3 classes: IFN-α, IFN-β and IFN-γ (Clemens and McNurlan, 1985; Virelizier and Arezana-Seisdedos, 1985; Zoon, 1987).

IFN-α is produced by null leucocytes and macrophages in response to viruses and is coded for by genes located on chromosome 9 in man. The

IFN-α family comprises more than 24 species that share >50% sequence homology. The mature protein(s) consists of 166 amino acids with an apparent molecular weights of 16-27kD. Most of the proteins are not glycosylated. The functional unit is a monomer.

IFN-β is produced by fibroblasts in response to viruses. Only one type of IFN-β has been isolated. The functional unit is a dimer, the monomer being a glycoprotein of 166 amino acids with an apparent molecular weight of 20kD. The IFN-β gene is located on chromosome 9 in man.

IFN-α and IFN-β share a common high affinity cell surface receptor (Branca and Baglioni, 1981; Tamm et al., 1987) with a dissociation constant of 20-500pM. As with many other ligands, the receptor-ligand complex is internalized via receptor-mediated endocytosis, where it is degraded and then released from the cell.

IFN-γ consists of multiple protein species produced by post-translational modifications of the product of a single gene located on chromosome 12 in man. It is a glycoprotein of 146 amino acids with an apparent molecular weight of 20-25kD. The functional unit is a tetramer. IFN-γ is produced by T cells in response to antigen or mitogen and is distinguished from the other interferons by being labile at pH 2. IFN-γ has a separate distinct cell-surface receptor (Branca and Baglioni, 1981).

Interferon inducers other than viruses include platelet-derived growth factor, tumour necrosis factor, colony stimulating factor 1 and

interleukin 1. The interferons are not species specific but have, instead, defined host ranges with some interferons being more active in some species than in others. They are antigenic only in heterologous hosts.

Their prime effects are to make cells resistant to virus infection, to inhibit cell proliferation, to modulate cell differentiation and to inhibit transformation (Tamm et al., 1987; Stewart, 1979). The interferons have been shown to activate several genes e.g.class I HLA genes are induced by all the interferons, class II HLA genes are induced predominantly by IFN-γ (Tamm et al., 1987).

IFN has been shown to regulate collagen synthesis by decreasing the levels of procollagen mRNA (Rosenbloom et al., 1984; Stephenson et al., 1985). IFN has also been shown to decrease fibroblast proliferation (Duncan and Berman, 1985). IFN-γ is the most potent inhibitor of collagen synthesis but IFN-α is also effective (Jimenez et al., 1984).

Tumour necrosis factor

Tumour necrosis factor (TNF) is mentioned for its functional similarity to IL-1. It is a 17kD protein produced by monocytes/macrophages (Cerami and Beutler, 1988). It is produced as a prohormone with an additional 76 amino acids on the amino-terminus. The gene for TNF is located on chromosome 6 in man (Cerami and Beutler, 1988).

Synthesis of TNF is controlled at the post-transcriptional level. The mRNA molecules have a consensus AU sequence which confers instability on the mRNA. This allows short bursts of intense activity before the mRNA is degraded and the activity decreases. TNF is species-preferential rather than species-specific. TNF is highly conserved and must, therefore, play some important role in the cell, presumably mediating resistance to a wide range of organisms and tumours.(Cerami and Beutler, 1988).

TNF has been shown to decrease collagen synthesis in bone (Bertolini et al., 1986) and to increase collagenase and prostaglandin E_2 synthesis in synovial cells and dermal fibroblasts (Dayer et al., 1985). It stimulates the growth of normal fibroblasts (Vilcek et al., 1986; Sugarman et al, 1985).

Many of the cytokines can act directly, as already shown, or indirectly by inducing the synthesis of other cytokines. Both TNF and IFNs, for example, increase IL-1 production by monocytes in response to lipopolysaccharide. (Philip and Epstein, 1986). Some of the effects of the cytokines are mediated by prostaglandin E_2 (Laato and Heino, 1988).

Prolonged exposure of fibroblasts to mononuclear cell products may alter the fibroblast phenotype. The synthesis of glycosaminoglycans (Worrall et al., 1986) and of prostaglandin E_2 (Korn, 1983) have been shown to be abnormal after prolonged exposure to mononuclear cell products - in all probability due to the selection of a subpopulation

of cells which produce high levels of glycosaminoglycans or are high producers of prostaglandin E_2. It has been suggested that this is responsible for the abnormal fibroblast phenotype seen in disease states such as progressive systemic sclerosis (Worrall et al., 1986; Korn, 1983).

Peptide Growth Factors

A number of peptide growth factors have recently been shown to influence the deposition of extracellular matrix and the function of mesenchymal cells. Transforming growth factor beta (TGF-β) and fibroblast growth factor (FGF) have particular relevance to the theme of this thesis.

Transforming Growth Factor-β

Transforming growth factor-β is a homodimeric protein with a monomeric molecular weight 25 kD (Sporn et al., 1987). First discovered for its ability to promote anchorage-independent growth of normal cells *in vitro*, it is produced by a wide variety of cells, both neoplastic and non-neoplastic, and is particularly abundant in platelets and bone. The receptors for TGF-β are universally and constitutively expressed. It is secreted in a latent form which is unable to bind to its receptor. It is most likely activated by the action of proteases which disrupt the quaternary structure.

There are two forms of TGF-β: TGF-β1, the original isolate from platelets and TGF-β2 which constitutes 15-20% of the total TGF-β. Their

functions are indistinguishable, but it appears that they may have different receptors. Both forms are highly conserved with total sequence homology between human, bovine and porcine species and only one amino acid substitution in the murine species.

TGF-β belongs to a larger gene family coding for molecules which all have growth regulatory properties. Other members include inhibin, activin and Mullerian substance (for review see Sporn et al., 1987).

The high degree of evolutionary conservation of TGF-β and the striking effects that it has in many experimental systems argue in favour of its having a critical role to play in development and function of the organism. The physiological role of TGF-β, however, is unknown and Sporn et al. (1987) suggest that TGF-β is a 'morphogenetic' substance that modulates responses to other growth factors and regulates cell growth.

It enhances the proliferation of cells of mesenchymal origin and inhibits the proliferation of cells of epithelial origin, endothelial cells and B and T lymphocytes. It stimulates the production of extracellular matrix by increasing the synthesis of matrix components and decreasing their degradation. It antagonises the mitogenic actions of epidermal growth factor, platelet-derived growth factor, fibroblast growth factor, insulin and insulin-like growth factor.

In a recent study on the role of TGF-β in the development of the mouse embryo, it was found that TGF-β is localized in mesenchyme and mesenchymally-derived tissues (Heine et al., 1987).

TGF-β is a potent desmoplastic agent (Raghow et al., 1987). It increases collagen and fibronectin synthesis in chicken, human and rat cells (Fine and Goldstein, 1987; Ignotz and Massague, 1986; Ignotz et al., 1987; Varga and Jimenez, 1986); it induces the formation of granulation tissue when injected into newborn mice (Roberts et al., 1986); it promotes wound healing (Sporn et al., 1983); and it is chemotactic for both fibroblasts and monocytes (Postlethwaite et al., 1987; Wahl et al., 1987).

The increased rates of protein synthesis induced by TGF-β are accompanied by increased levels of mRNA. This could be due either to increased stability of the mRNA (Raghow et al., 1987) or due to increased transcriptional activity. Rossi et al. (1988) have recently shown that TGF-β increases the activity of the promoter region of the collagen gene. This is mediated by binding of nuclear factor 1: a DNA-binding protein that activates eukaryotic gene transcription (Jones et al., 1987).

TGF-β also increases net matrix accumulation by decreasing degradation of the matrix (Laiho et al., 1986). It achieves this in two ways. Firstly, it increases the production of protease inhibitors e.g. tissue inhibitor of metalloproteinases. Secondly, it decreases the production of the proteases themselves e.g. plasminogen activator.

Since TGF-β is chemotactic for monocytes, some of its effects may be mediated by these cells that can be activated to produce other cytokines such as IL-1.

TGF-β is one of the lymphokines produced by activated T cells (Kehrl et al., 1986) and may be responsible for some of the fibrogenic activity found in mononuclear cell supernatants. Antibodies to TGF-β partially abolish the stimulatory activity of such supernatants (Roberts et al., 1986).

Fibroblast Growth Factors

There are two forms of fibroblast growth factor (FGF): acidic FGF and basic FGF, which share 55% total sequence homology and a strong affinity for binding to heparin. Both are potent mitogens and differentiation factors for a wide variety of mesoderm- and neuroectoderm-derived cells (for review see Gospodarowicz, 1987).

Basic FGF (bFGF) is an evolutionary well-conserved single chain polypeptide with an apparent molecular weight of 16 kilodaltons. It is encoded by a gene on chromosome 4 in man and is found in a wide variety of tissues.

Acidic FGF (aFGF) is a less well-conserved single chain polypeptide, with an apparent molecular weight of 14-15 kilodaltons and has been found only in brain, retina, bone matrix and osteosarcoma. Its action is augmented by heparin (Uhlrich et al., 1986).

Both FGFs bind to the same high affinity plasma membrane receptors which are found on all cell types capable of responding to FGF (Gospodarowicz, 1987). In culture, bFGF is associated mainly with cells and basement membranes and is not released into the medium (Klagsbrun et al., 1986; Vlodavsky et al., 1987). As with IL-1, there is an apparent absence of a secretory-signal sequence in the bFGF protein (Abraham et al., 1986) and it is uncertain how FGF is transported to the cell exterior.

In vivo, they induce the formation of highly vascularised granulation tissue in sponges implanted subcutaneously in the rat (Davidson et al., 1985). Here the increase in collagen accumulation is due to an increase in the number of mesenchymal cells rather than to an increase in the cellular rate of collagen synthesis.

Other Growth Factors

Other growth factors shown to increase collagen production are insulin (Kjellstrom and Malmquist, 1984) and platelet-derived growth factor (PDGF)(Ross et al., 1986). PDGF stimulates both general protein synthesis and collagen synthesis. It also stimulates collagenase synthesis. Thus, PDGF may be important in (i) wound healing, by inducing fibroblast migration, proliferation and matrix synthesis; (ii) in neoplasia, by inducing adjacent stromal cells to secrete matrix components; and (iii) in bone marrow fibrosis, by inducing marrow stromal cells to produce excess connective tissue. Epidermal growth factor, like FGF, increases collagen accumulation by increasing

the number of cells producing collagen rather than increasing collagen synthesis (Laato et al., 1987).

Drugs

Glucocorticoids

It is well known that topical treatment with glucocorticoids leads to atrophy of the skin (Adamson, 1982; Cockayne et al., 1986) associated with a decrease in collagen production that is attributable either to a selective decrease in the activity of prolyl hydroxylase (Adamson, 1982) or due to a decrease in procollagen mRNA levels (Cockayne et al., 1986; Raghow et al., 1986; Weiner et al., 1987). The decrease in mRNA levels is due either to changes in the rate of transcription of the collagen genes (Weiner et al., 1987) or to an increased turnover of the procollagen mRNA (Raghow et al., 1986). The glucocorticoid effect is receptor mediated: antagonists block the inhibition of collagen synthesis, whilst agonists enhance the inhibition (Cockayne et al., 1986).

Retinoids

Retinoids also inhibit collagen synthesis with a corresponding decrease in procollagen mRNA levels (Daly and Weston, 1986; Hein et al., 1984; Ohta and Uitto, 1987).

Catecholamines

The β-agonists, isoproterenol and epinephrine, inhibit collagen production by increasing the intracellular cAMP levels. This inhibition is blocked by the β-blocker, propanalol (Berg et al., 1981).

Bleomycin

The bleomycins are a family of chemotherapeutic proteins used in the treatment of malignancies. Their major toxic side effect is pulmonary fibrosis, caused by an increase in collagen accumulation (Sterling et al., 1983). The increase in collagen production is not paralleled by an increase in procollagen mRNA levels. Instead there is a repartitioning of the available mRNA between the nucleus, the polysomes and the cytoplasm. Nuclear and cytoplasmic mRNA levels increase.

Bleomycin-induced fibrosis in hamsters is self-limiting (Clark and Greenberg, 1987; Clark et al., 1983) with a transient rise in collagen production that soon returns to normal. These effects are mediated by alveolar macrophages that produce factors that both stimulate and inhibit collagen synthesis so that at any one time the net result reflects the balance between these opposing influences. Some of the effects of bleomycin may thus be mediated via secondary interactions with other cells.

Mast Cells

The two host cells most commonly associated with neoplasia are the macrophage and the mast cell. I have already discussed the implications of macrophage involvement and its possible role in the induction of desmoplasia. The mast cell, too, is associated with fibrosis.

The mast cell is a leucocyte that contains numerous metachromatic granules and is found in connective tissue, particularly where potentially noxious substances can enter the body (Pepys and Edwards, 1979). The granules contain a number of mediators: vasoactive/smooth muscle reactive mediators e.g. histamine; chemotactic factors; proteoglycans e.g. heparin; and enzymes e.g. chymase. They have receptors for the Fc portion of IgE and for C3b and can be triggered to release the contents of their granules by IgE-antigen interactions, anaphylatoxins, enzymes, ionophores and polycations. They provoke a biphasic inflammatory response. The first phase, a vasodilatory phase and the second, a cellular phase. If these responses continue unchecked, they can lead to tissue damage and fibrosis.

Mast cells have been associated with pulmonary fibrosis, wound healing, chronic graft-versus-host disease and scleroderma (Claman, 1985). It appears that mast cells through their mediators, histamine and heparin, stimulate fibroblast growth and not fibroblast collagen synthesis. Heparin has affinity for several growth factors including

both basic and acidic fibroblast growth factor (Folkman, 1986). Heparin also stimulates angiogenesis and the myofibroblasts that form part of this response may lead to fibrosis (Beranek and Clevy, 1985).

Recent experiments with TSK mice have supported the notion of mast cell involvement in fibrosis in vivo. These animals have a genetically transmitted connective tissue disease analogous to scleroderma. If they are treated with disodium chromoglycate, an inhibitor of mast cell degranulation, a reduction of both the number of degranulated mast cells in the skin and the thickness of the skin is observed (Walker et al., 1987).

CHAPTER I

GROWTH AND CHARACTERISTICS OF UCT-MEL 7 DERIVED TUMOURS IN THE NUDE MOUSE

The successful growth of a human tumour as a xenograft in an athymic nude mouse was first reported in 1969 (Rygaard and Povlsen, 1969), and since that time this animal model has been used extensively to study malignant cell growth (Freedman and Shin, 1978; Giovanella and Stehlin, 1974; Houchens and Ovejera, 1978) and the way in which the neoplastic phenotype is expressed or can be modulated. Invasion and metastasis (De Vore et al., 1980; Fidler et al., 1978; Thorgeirsson, 1985), angiogenesis (Folkman, 1975), and the complex interactions between host and tumour are all aspects of neoplasia that are best studied *in vivo* and the nude mouse has provided a very useful approximation to the original human situation.

Tumours that develop following inoculation of malignant cells into an athymic mouse usually grow relentlessly and exponentially. If the tumour is allowed to remain *in situ* the host usually dies shortly after the growth phase is established.

My concern in this thesis is with the unusual tumours that developed when cells from a human melanoma cell line, UCT-Mel 7 (Hoal-van Helden et al., 1986) were inoculated into nude mice. These tumours differed from the usual melanoma xenografts in a number of interesting respects. They showed an atypical growth pattern; they elicited a

marked desmoplastic response in the host; and they occasionally gave rise to murine tumours.

MATERIALS AND METHODS

Melanoma cells

The UCT-Mel 7 cell line was established from the femoral lymph node of a 52 year old African woman who presented, initially, with a chronic ulcer on the left heel. This was removed and diagnosed as a malignant melanoma, Clark's level V. Two months later a left femoral gland dissection revealed nodes that were infiltrated with tumour tissue.

The node that I received had a firm, rubbery consistency and showed, on histological examination, the presence of secondary malignant melanoma with a spindle cell morphology and minimal melanin production (Fig. 1).

The sample was minced finely and the fragments digested with trypsin, and collagenase. Sparse cultures of slowly growing cells were obtained from the enzymatically treated fragments plated in RPMI-10. These were passaged at confluence until a non-pigmented, continuous cell line was established. The cells grew as an adherent monolayer with a pronounced spindle cell morphology (Fig. 2). Several other melanoma cell lines that had been established in this laboratory were also available to me and these provided interesting comparison with the UCT-Mel 7 line. These lines have been well described in published

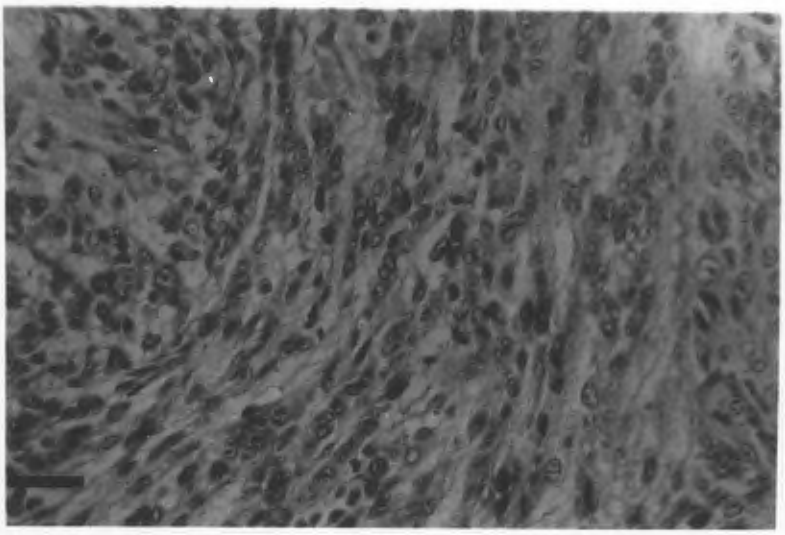


FIGURE 1

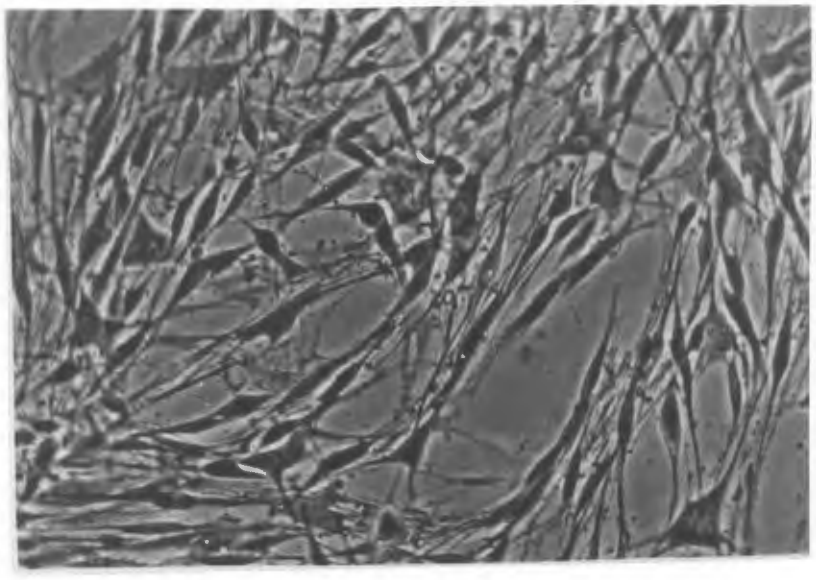
Histological appearance of original UCT-Mel 7 tumour

The photomicrograph shows the histological appearance of a fragment of the original melanoma-containing femoral lymph node from which the UCT-Mel 7 line was established. It shows a malignant melanoma with a spindle cell morphology and minimal melanin production.

The scale marker represents 30μm.

FIGURE 2

A



B

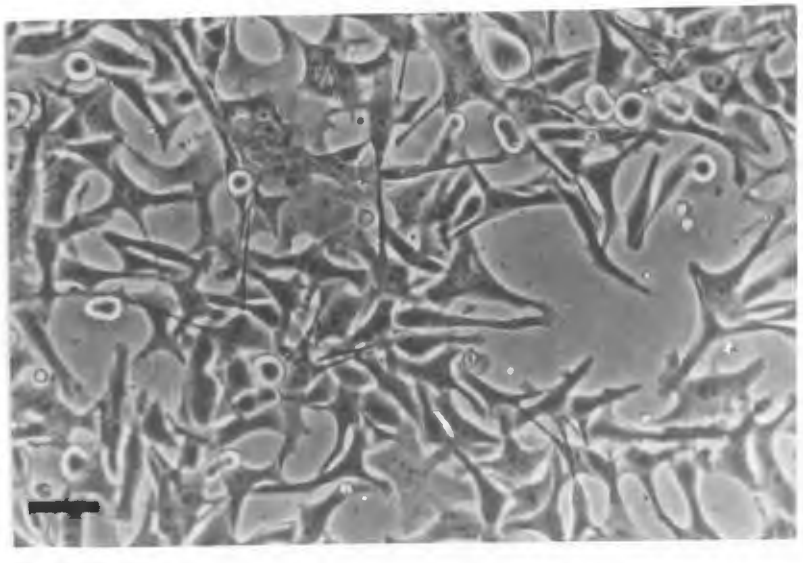


FIGURE 2

FIGURE 2

Morphological appearance of two melanoma cell lines in culture.

The photomicrographs emphasise the different morphologies of the adherent monolayers of melanoma cell lines in culture.

Fig. 2a. UCT-Mel 7 cells grew with a pronounced spindle cell morphology.

Fig. 2b. UCT-Mel 1 cells showing triangular/dendritic morphology more typical of melanomas.

The scale marker represents 50μm.

reports (Hoal-van Helden et al., 1986). I summarize their general characteristics in Table 1.

Cultures were maintained using standard techniques of tissue culture (Freshney, 1987) in RPMI supplemented with antibiotics and 10% foetal calf serum. Stocks of cells at different passage numbers were frozen and stored in liquid nitrogen (Farrant et al., 1974).

Nude mice

Athymic nude mice of N:NIH (S) II - nu/nu strain (Azar et al., 1980) were bred in the UCT Animal Unit from breeding stocks imported in June 1980 and provided by Dr. B.C. Giovanella of the Cancer Research Laboratory, St. Joseph's Hospital, Houston, Texas, U.S.A. This strain has combined immunodeficiency i.e. in addition to the thymic hypoplasia found in the original strain of nu/nu mice, both T-cell and B-cell zones of the lymph nodes and spleen are depleted of lymphocytes. The colony is maintained by breeding pairs of the heterozygous nu/+ females and homozygous nu/nu males. The homozygous female offspring were used for experiments.

The mice were handled under stringent sterile conditions at all times. All tumour cell inoculations and other manipulations were conducted in a laminar flow hood by personnel clothed in sterile gowns and wearing sterile rubber gloves. Athymic mice kept under these conditions thrived and had lifespans comparable to those of normal mice.

TABLE 1

TABLE 1
Characteristics of the melanoma cell lines

	UCT-Mel 1	UCT-Mel 2	UCT-Mel 3	UCT-Mel 4	UCT-Mel 5	UCT-Mel 6	UCT-Mel 7
Doubling time (hr)[a]	41	50	50	52	58	33	95
Morphology [b]	Triangular/ Dendritic	Dendritic	Triangular/ Dendritic	Polygonal	Triangular/ Dendritic	Epithelioid/ Spindle Cell	Spindle Cell
Pigmentation[c]	+	+	-	-	-	-	-
Growth in soft agar(%)[d]	57.4	63.2	53.8	0	18.6	0	1.8
Serum dependence (%)[e]	0	0	10	10	10	10	10
Growth in nude mice[f]	+	+	+	+	+	-	+
Metastasis in nude mice(%)[g]	6 Lung	12 Lung	100 Lung Gonads Kidney Liver Intestine	10 Lung	14 Lung	-	0

Footnotes to Table 1

a) Doubling times were taken from growth curves constructed by seeding the cells at a low density (usually 10^5 cells/35 mm dish) and feeding with fresh medium every 48 hours. Cells were detached at 48 h intervals and counted.

b) To illustrate the difference between the cellular morphology of UCT-Mel 7 and the more typical melanomas, phase-contrast photomicrographs of UCT-Mel 7 and UCT-Mel 1 are presented in Fig. 2a and 2b, respectively.

c) The melanin contents of the original biopsy specimens and pelleted cells were assessed visually or by measuring the cellular tyrosinase content.

d) Cells were plated in 0.33% Agar in RPMI-10. Colonies were scored after 14 days and expressed as a percentage of the cells plated.

e) Minimum concentration of serum required to maintain active proliferation in vitro.

f) Nude mice received subcutaneous inocula of melanoma cells varying in number from $1x10^6$ to $5x10^7$. Animals were scored weekly for up to 6 months, for evidence of tumour formation at the site of inoculation.

g) Metastatic spread was evaluated by post mortem examination after excision of the primary tumour and a prolonged (usually 6-10 months) period of observation (cf Table 2).

Inoculation into nude mice

Cells to be inoculated into the mice were released from the tissue culture dish with 0.25% trypsin containing 0.02% EDTA in a buffered salt solution. The trypsin was neutralized by the addition of foetal calf serum to a final concentration of 5%, and the cells were then pelleted, washed once with serum-free medium, and adjusted to give the requisite number of cells in an inoculum of 0.1 ml.

The cells were injected subcutaneously into the inter-scapular region of 6-8 week old mice. The animals were subsequently examined at weekly intervals when tumour growth was recorded.
The tumour volume was calculated as the product of 3 major diameters.

When called for by the experimental protocol, tumours were removed under light ether anaesthesia and the skin incision closed with surgical clips that were removed after one week. Alternatively, the tumours were removed after killing the animals.

Each tumour was divided into several representative portions. One of these was placed in buffered formol saline for histology. The other fragments were variously used for re-inoculation, re-establishment in culture, biochemical analysis or cryopreservation (Farrant et al., 1974).

Analysis of the tumours

a) Histology of the Tumours

Formalin-fixed samples were embedded in paraffin, sectioned and stained according to conventional histological techniques (Mayers' haematoxylin and eosin). Sections of the tumours were stained for reticulin according to the method of Gordon and Sweets (1936).

b) Hydroxyproline Content of the Tumours

The hydroxyproline content of the tumours was determined using the method of Hutterer and Singer (1960) in which tissues were hydrolysed in 6N HCl, evaporated to dryness under vacuum and the residue dissolved in distilled water. Samples so obtained were assayed for hydroxyproline content by reading the absorbance at 500nm and 560nm after the addition of p-dimethylaminobenzaldehyde and referred to a standard curve.

c) Phosphoglucoisomerase Analysis

To determine the relative contributions of human and murine cellular elements to tumours that were obtained, lysates were prepared by homogenizing the tumours in 2 volumes of 0.1M sodium phosphate pH 7.4. These were then analysed for the phosphoglucoisomerase (PGI) enzymes they contained using standard techniques of starch gel electrophoresis and zymography (Siciliano and Shaw, 1976; Harris and Hopkinson, 1976).

Human and murine PGI isoenzymes were readily distinguishable by differences in their electrophoretic mobilities.

Chemotaxis

Chemotaxis was performed using the standard micropore filter assay with sawn-off tuberculin-syringe barrels as the upper chamber (Wilkinson, 1982). Peritoneal cells were harvested from BALB/c female mice and used at a concentration of $1x10^6$ cells/ml. The filters used were Sartorius 8 μm filters. Chemotaxis was measured over 5 hours at 37°C using the leading front method of scoring. A 0.5% solution of sodium caseinate (Merck 2242) was used as the positive control.

Superoxide anion production

Superoxide anion secretion was assayed by the superoxide dismutase-inhibitable reduction of ferricytochrome c. (Johnston et al., 1978). UCT-Mel 7 cells and foreskin fibroblasts were plated at $1x10^5$ cells/16mm well in 1 ml Hanks balanced salt solution without phenol red (Gibco 076-1201) supplemented with 5.5mM glucose, (BDH 10117), 25 mM Hepes (Boehringer Mannheim 223778), MEM amino acid mix (50x conc.; Gibco 043-1130H), L-glutamine (Sigma G3126) and MEM vitamin mix (100x conc.; Gibco 043-1120H). Thioglycollate-elicited mouse peritoneal macrophages were harvested by peritoneal lavage of BALB/c mice and plated at $8x10^5$ cells/16mm well. Non-adherent cells were removed by washing after 2 hours incubation.

At the start of the experiment ferricytochrome c (Sigma C-2506) was added to give a final concentration of 10^{-5}M. To some assay wells

was added superoxide dismutase (Oxinorm) at a final concentration of 150U/ml and to other wells tetradecanoyl phorbol acetate (PMA; Consolidated Midland Corporation) at a final concentration of 10 ng/ml.

The cells were incubated at 37°C in 5% CO_2 - 95% air. At the indicated times, the reaction mixture was removed from the wells, centrifuged at 3000g in an Eppendorf microcentrifuge and the optical density measured at 550 nm. The concentration of cytochrome c reduced was determined using the equation $\Delta E_{550nm} = 21 mM^{-1}cm^{-1}$.

At the end of the experiment, the cells were digested with 1M NaOH and 10% Na deoxycholate (O/N at 37°C) and the protein content of the digest determined by the method of Lowry et al. (1957) using BSA as standard.

RESULTS

In most cases, malignant melanoma cells injected into nude mice give rise to tumours that develop at the site of inoculation and subsequently show a characteristic pattern of growth in which there is an initial latent period, during which the tumour is growing but imperceptible, followed by overt exponential growth. A typical example of this behaviour was shown by UCT-Mel 1 (Fig. 3) that grew exponentially with a doubling time of 4.6 days. UCT-Mel 7, however, consistently showed a different pattern of growth. After a period of

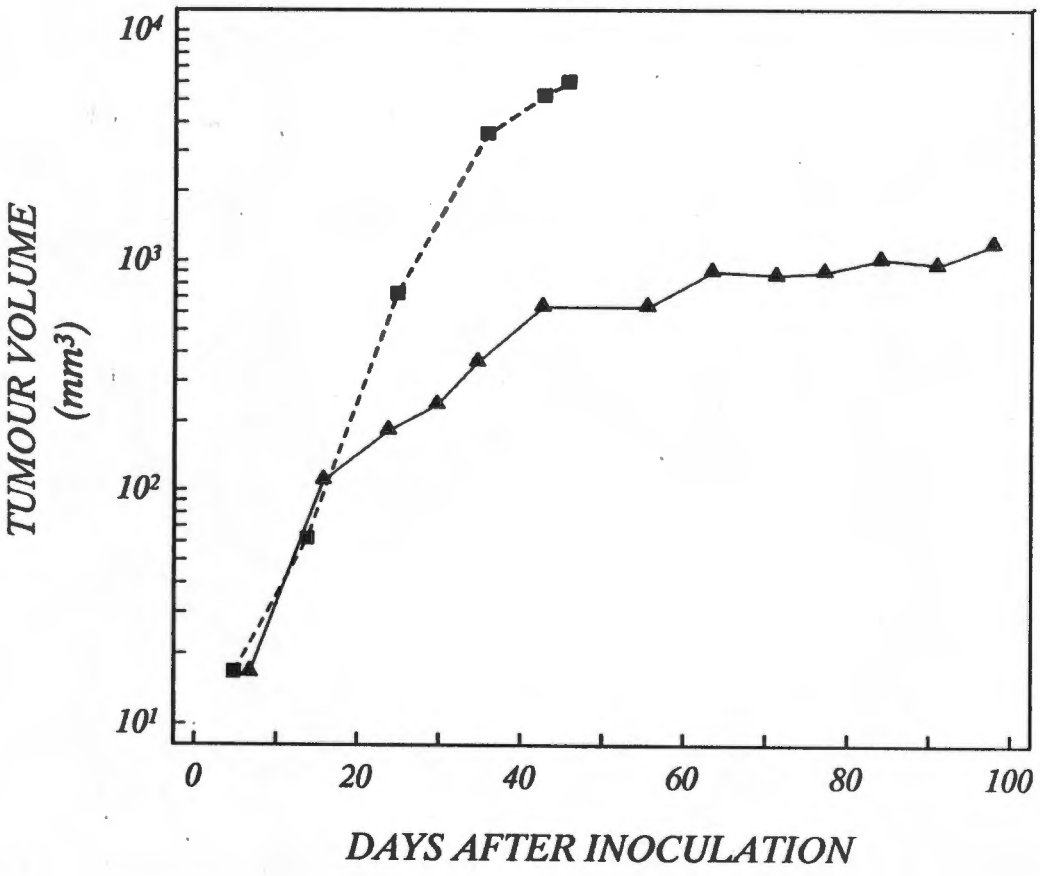


FIGURE 3

Growth of human melanomas in nude mice: comparison of UCT-Mel 1 and UCT-Mel 7 derived tumours.

The figure shows tumour volumes as a function of time after the subcutaneous injection of $5x10^6$ cells into each of 2 mice.

The tumour that developed following injection of UCT-Mel 1 cells (■----■) showed the usual pattern of exponential growth following a short latent period.
The UCT-Mel 7 derived tumour (▲——▲) grew exponentially for a short while after which growth was arrested at a plateau.

These results are typical of many similar experiments and record the growth of UCT-Mel 7 derived tumours during the first 100 days. Subsequent growth kinetics of UCT-Mel 7 derived tumours are shown in the figures that follow.

early proliferation, growth ceased at a plateau that was maintained for 2 to 3 months.

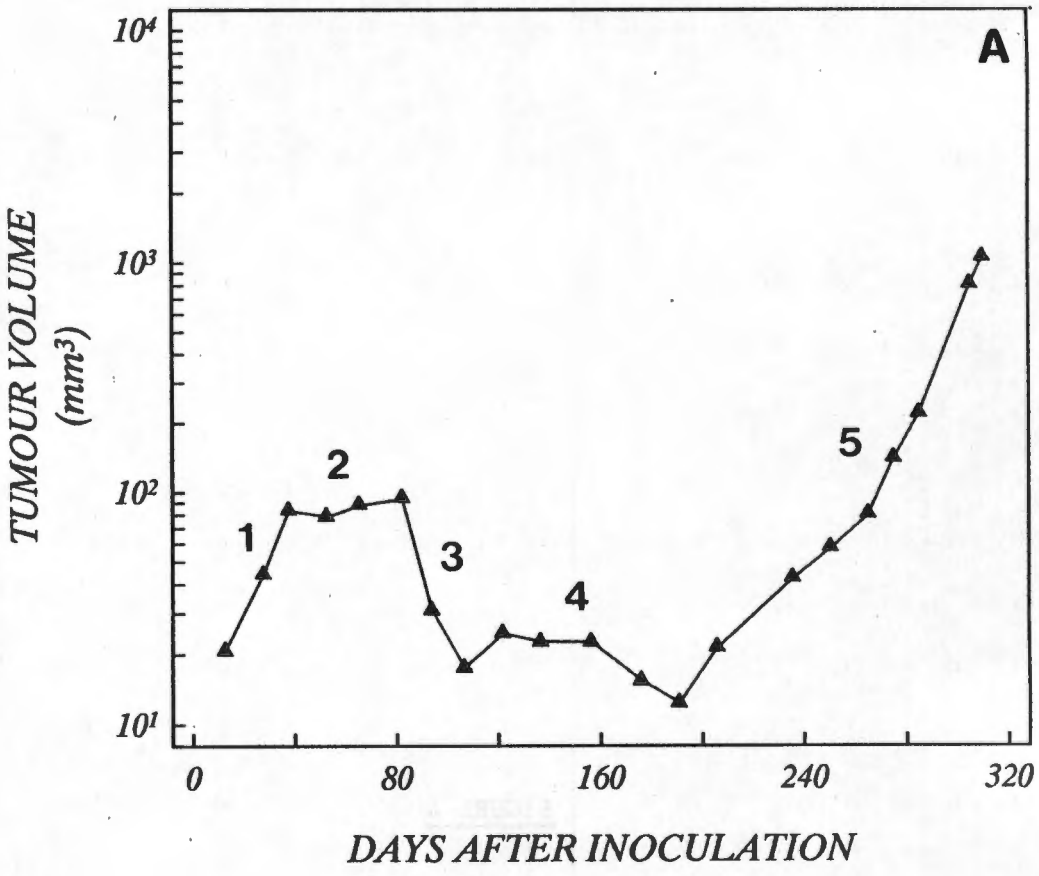
This period of dormancy was followed by a period of regression during which time the tumour diminished in size. After a further dormant spell, and by now 4 to 7 months after the initial inoculation, the tumour began to grow again, this time showing simple exponential kinetics (Fig. 4a). Tumours that emerged from the period of dormancy grew much more aggressively than the initial tumour.

The growth pattern could thus be divided into 5 sequential phases:

1. a growth phase;
2. a plateau phase;
3. a phase of regression;
4. a dormant phase; and finally
5. a phase of exponential growth.

Not all tumours showed all 5 phases of growth. Some entered the second phase of exponential growth without regressing. Others regressed and then grew exponentially without the second dormant phase (Fig. 4b). Once the tumours had entered phase 5, however, they all grew with remarkably similar growth kinetics and, if they were removed at this stage and reimplanted into fresh recipients, the tumours that resulted grew exponentially without a dormant phase and with similar <u>in vivo</u> doubling times (Fig. 5). For the sake of descriptive

FIGURE 4


A
10^4
10^3
10^2
10^1
TUMOUR VOLUME
(mm^3)
1
2
3
4
5
0
80
160
240
320
DAYS AFTER INOCULATION

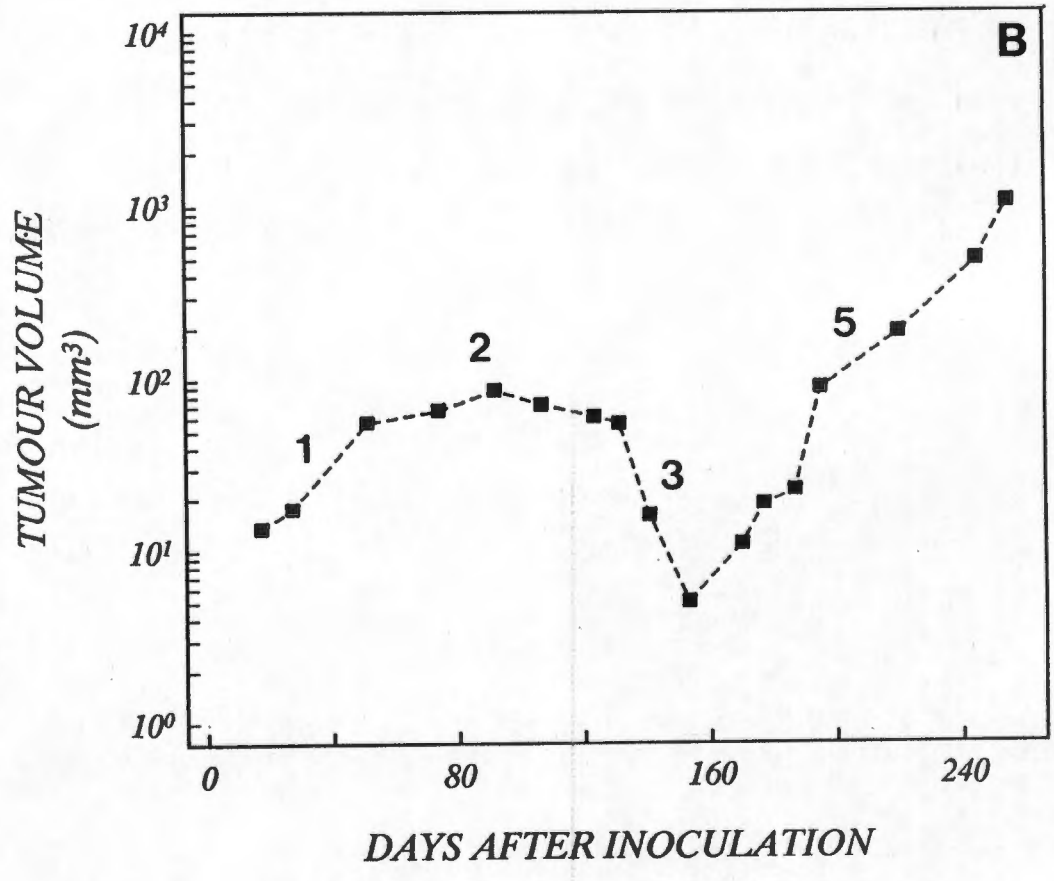

B
10^4
10^3
10^2
10^1
10^0
TUMOUR VOLUME
(mm^3)
1
2
3
5
0
80
160
240
DAYS AFTER INOCULATION

FIGURE 4

FIGURE 4

The phasic growth pattern of UCT-Mel 7 tumours in nude mice.

These two figures depict typical growth patterns observed when each of two mice received injections of $5x10^6$ UCT-Mel 7 cells taken from *in vitro* passage number 30.

Fig. 4a shows the typical phasic growth patterns referred to in the text in which an initial growth phase (1) was followed by a plateau phase (2) during which time no increase in tumour size occurred. This was followed subsequently by a phase of tumour regression (3), a period of dormancy (4) and, finally, an exponentially growth phase (5).

Fig. 4b shows the variation in the growth pattern that was occasionally observed. The exponential phase (5) followed the regression phase (3) without a preceding dormant phase (4).

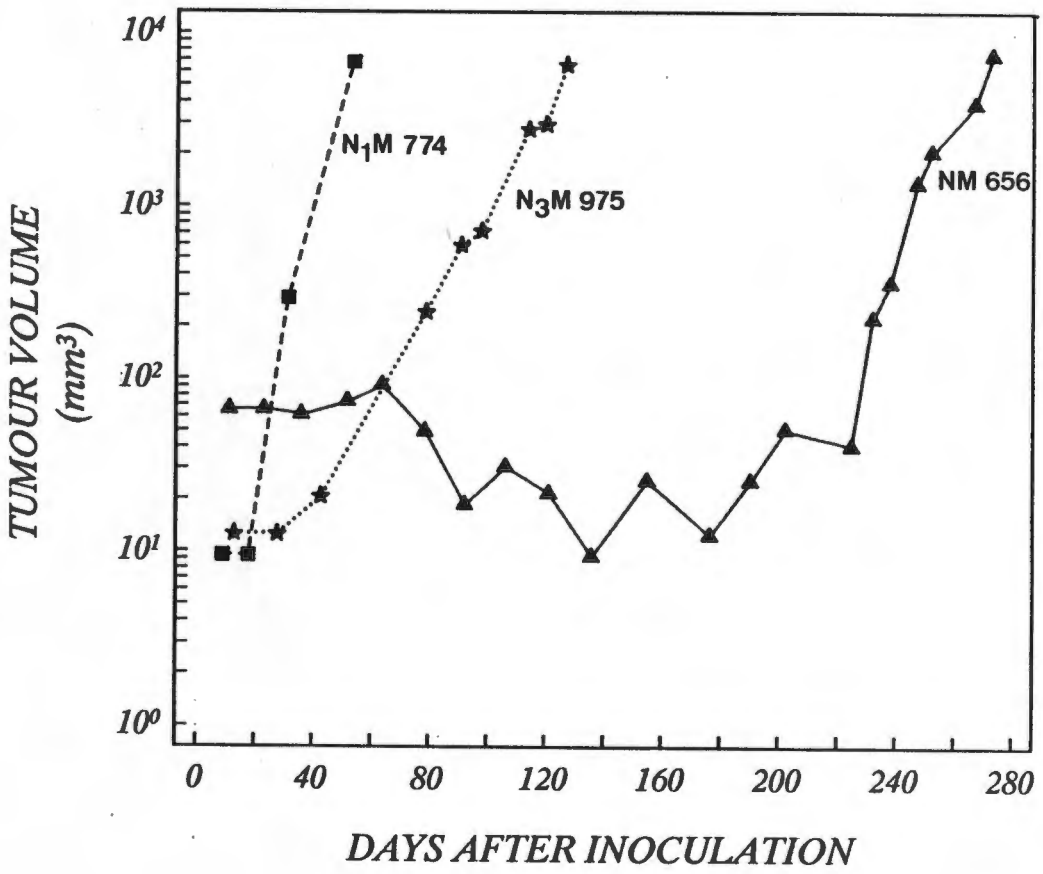


FIGURE 5

Growth patterns of UCT-Mel 7 tumours derived from fragments of tumours passaged in vivo.

The figure shows the growth patterns of tumours derived either from inoculation of $5x10^6$ UCT-Mel 7 cells or from subcutaneous implantation of tumour fragments taken from tumour-bearing mice.

The original tumour (NM 656), that developed from UCT-Mel 7 cells (▲——▲) showed the phasic pattern of growth described previously. The tumour (N_1M 774) that grew from fragments of NM 656 taken at 273 days and that (N_3M 975) that developed from a second in vivo passage of N_1M, no longer showed the phasic pattern of the parent NM 656, but grew exponentially, without a "plateau" phase.

convenience I refer to phase 5 tumours that were passaged *in vivo* as "5P" tumours.

The pattern of growth that was observed depended upon the number of *in vitro* passages that had elapsed since the establishment of the primary cultures. Cells derived from the 8th *in vitro* passage gave rise to tumours that grew exponentially without a plateau phase whereas cells from cultures that had been passaged 30 times, gave rise to tumours that showed the phasic growth pattern that I have described. Tumours arising from cells which had been passaged 56 times, showed a prolonged period of dormancy with eventual exponential growth (Fig. 6).

It was of interest to note the paradoxical inverse relationship between growth rates *in vivo* and the *in vitro* proliferative rates (Fig. 7). The tumours derived from *in vitro* 8th passage cultures had an *in vivo* doubling time of 65 days and an *in vitro* doubling time of 103 hrs. On the other hand, cells from an *in vitro* passage 56 gave rise to tumours which grew slowly in the mouse yet had an *in vitro* doubling time of only 47 hours. Similarly, the 5P tumours that were serially transplanted into fresh recipient hosts, had short *in vivo* doubling times yet proliferated very slowly *in vitro*.

Histological examination of the tumours removed in phase 2 showed an intense desmoplastic response with heavy staining for reticulin. UCT-Mel 7 tumours had an extensive reticulin fibre network (Fig. 8) whereas other melanomas showed reticulin staining that was either

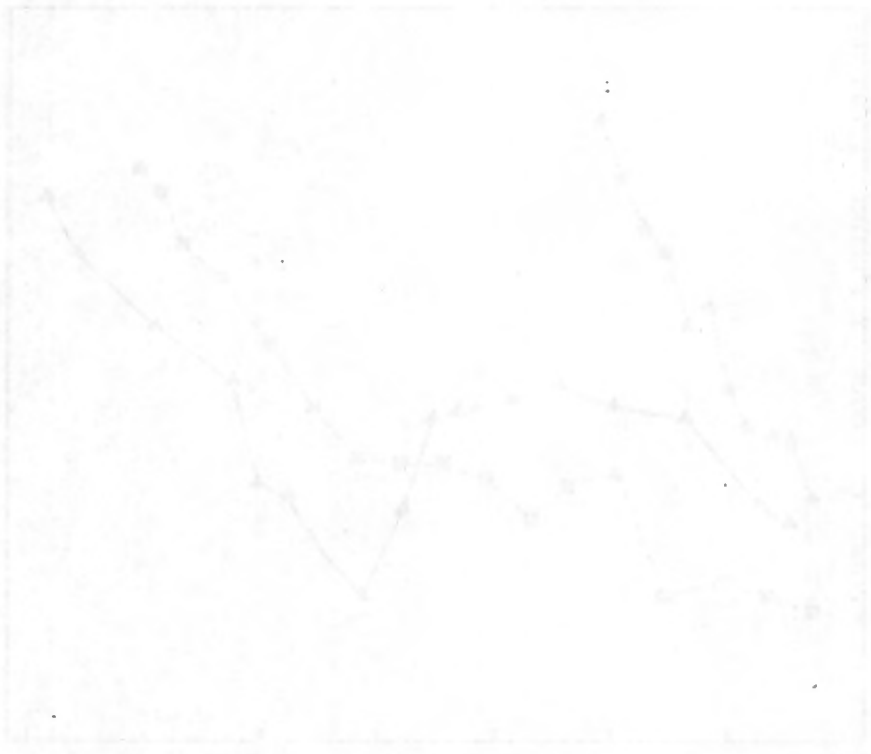


FIGURE 6

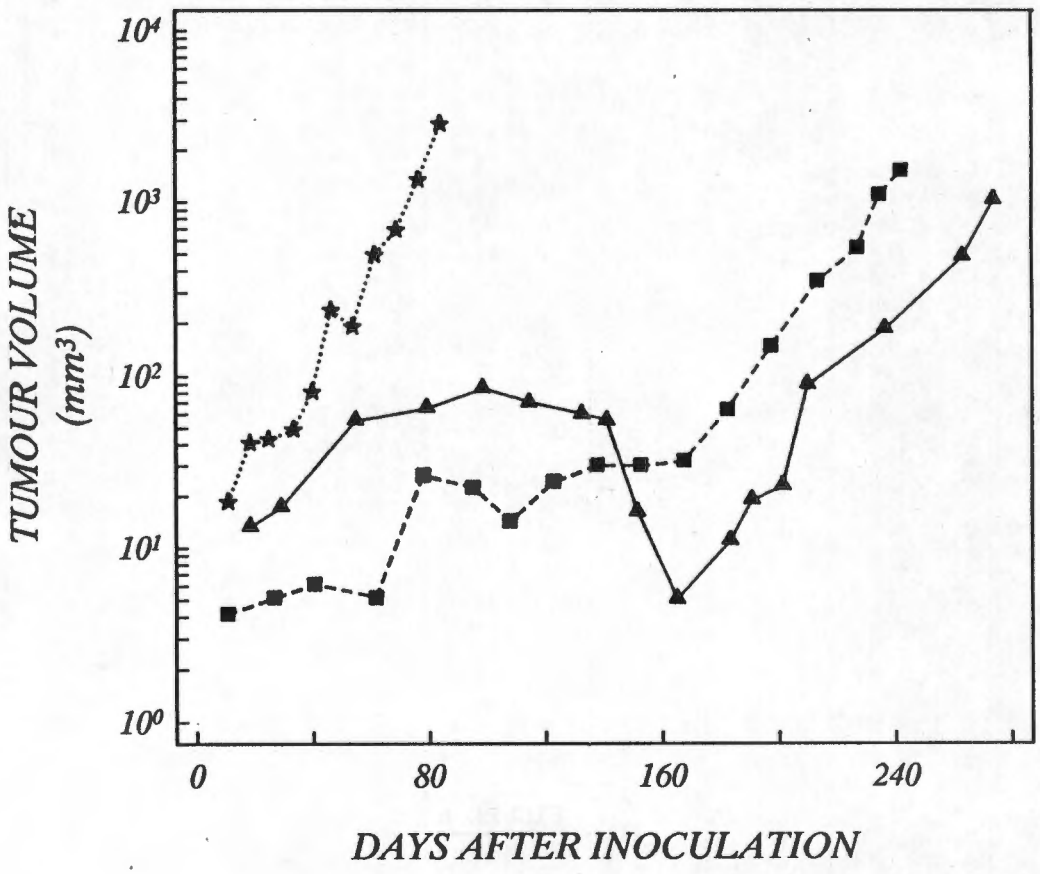

TUMOUR VOLUME
(mm³)
10⁴
10³
10²
10¹
10⁰
0
80
160
240
DAYS AFTER INOCULATION

FIGURE 6

FIGURE 6

In vivo growth patterns of tumours derived from different in vitro passages of UCT-Mel 7.

Three mice received inocula of $1x10^6$ UCT-Mel 7 cells of differing in vitro passage number. The tumours that developed were measured and then plotted as a function of time.

Cells from in vitro passage number 8 (✱·····✱) gave rise to a tumour that showed exponential growth from the start without any plateau phase.

The tumour derived from cells, from in vitro passage number 29 (▲——▲) showed a period of exponential growth, a plateau phase, a phase of regression and, subsequently, exponential growth.

An inoculum of cells from in vitro passage number 56 (■----■) developed into a tumour which grew exponentially following a prolonged lag phase.

The growth patterns depicted in this figure are representative of those observed in many other experiments. For the sake of clarity and brevity I present only three typical cases.

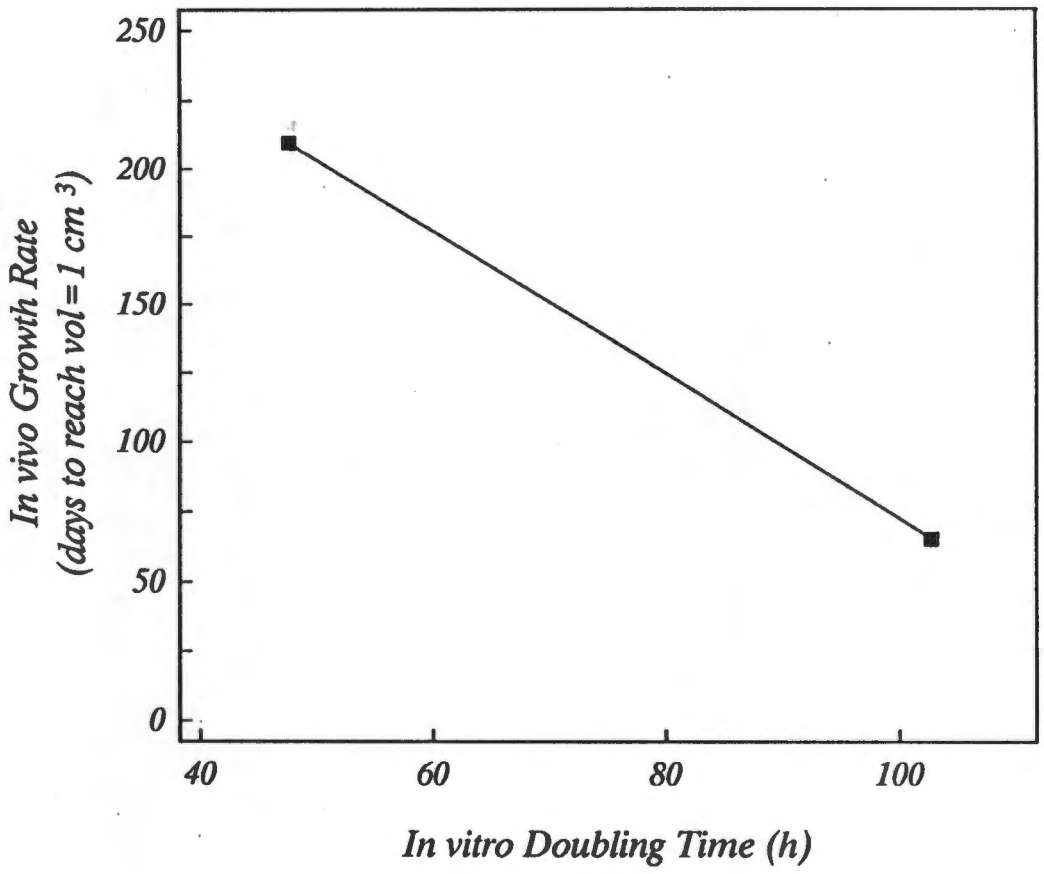


FIGURE 7

Inverse relationship between in vivo and in vitro growth rates

Tumours derived from 8th passage UCT-Mel 7 cells, which had an in vitro doubling time of 103 hours proliferated in vivo much more rapidly than tumours derived from 56th passage cells, which had an in vitro doubling time of 47 hrs.

In vivo growth was assessed as the time taken for the tumour to attain a volume of $1cm^3$.

In vitro proliferation was assessed as the time taken for the number of cells in the dish to double.

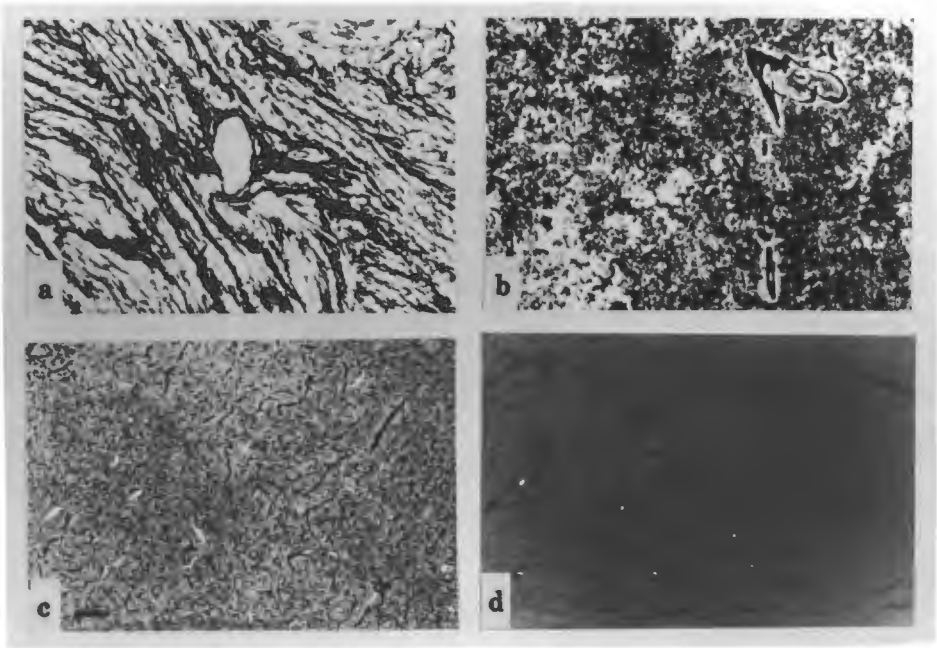


FIGURE 8

Melanomas excised from nude mice and stained for reticulin

Histological sections of tumours removed from nude mice and stained for reticulin. Scale marker = 60μm.

a) UCT-Mel 7, removed during phase 2, showing abundant reticulin arranged in a swirling pattern surrounding individual cells.

b) UCT-Mel 1, showing much less reticulin which appeared to be mainly perivascular.

c) UCT-Mel 2, showing virtually no reticulin.

d) UCT-Mel 7, removed during phase 5. Reticulin fibre formation is virtually absent.

perivascular or very scant. UCT-Mel 7-derived tumours that were removed during phase 5 did not contain detectable reticulin.

The histological assessment of collagen content was confirmed by chemical estimation of the hydroxyproline content of the tumours. UCT-Mel 7 tumours contained an average of 83.3 (± 9.8 sem) ng hydroxyproline per μg of cellular protein. The average hydroxyproline contents of the other melanomas that I examined ranged from 2.4 to 4.5 ng/μg cellular protein (Fig. 9).

The collagen content of the UCT-Mel 7 tumours varied at the different phases of growth (Fig. 10). Phase 2 tumours (i.e. those that showed a desmoplastic response) had high levels of hydoxyproline whereas the hydroxyproline content of phase 5 and 5P tumours was negligible.

Cultures of tumours that had been excised from the mice during phase 2 or phase 5 invariably comprised human cells that were visibly contaminated with murine fibroblasts and macrophage-like cells (Fig. 11).

Electrophoretic analyses of phosphoglucoisomerase (PGI) isoenzymes present in cell lysates provided a more definitive and quantitative estimate of the relative contribution of murine and human elements present in a tumour mass or in a mixed culture derived from a tumour (Fig. 12).

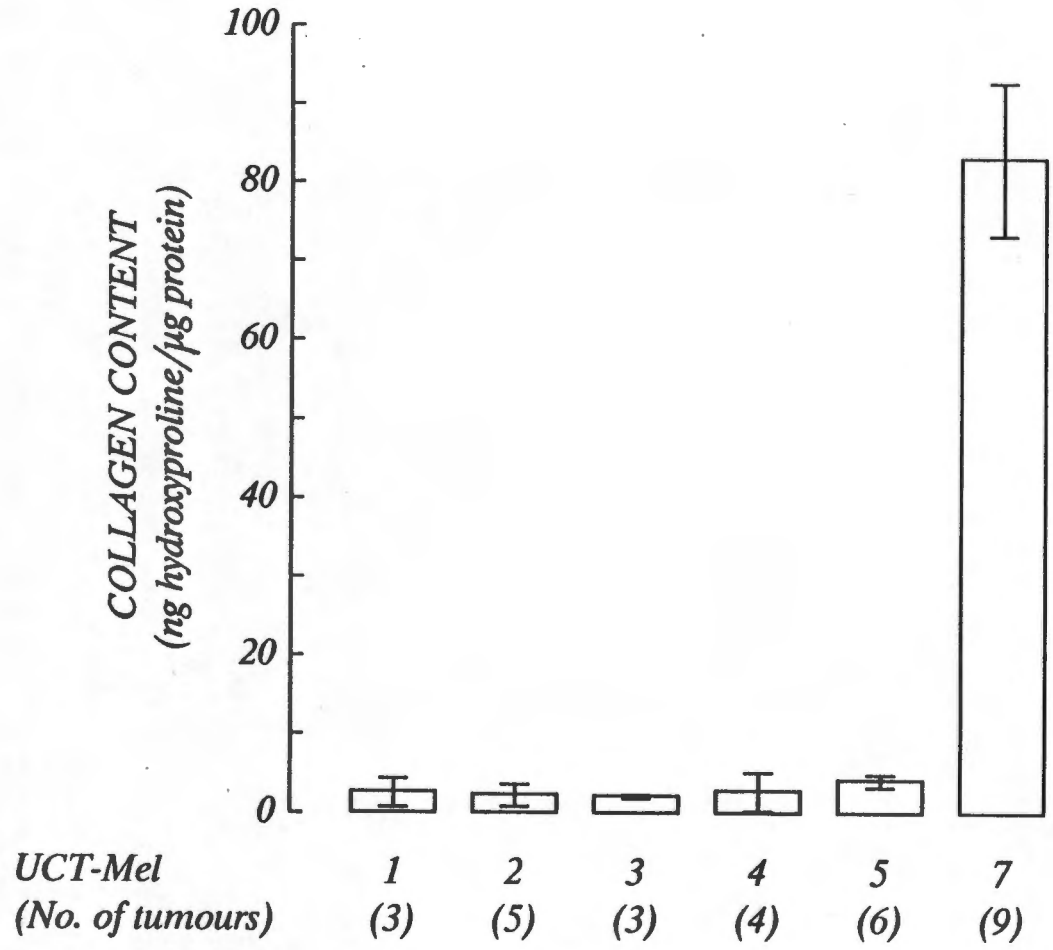


FIGURE 9

Collagen content of melanomas excised from nude mice

Tumours derived from different melanoma cell lines were excised and hydrolysed. The hydroxyproline content of the hydrolysates was measured and expressed in terms of total protein in the sample. Error bars represent ± 1 sem. The number of tumours assayed to provide the average values shown are given in parentheses below each bar.

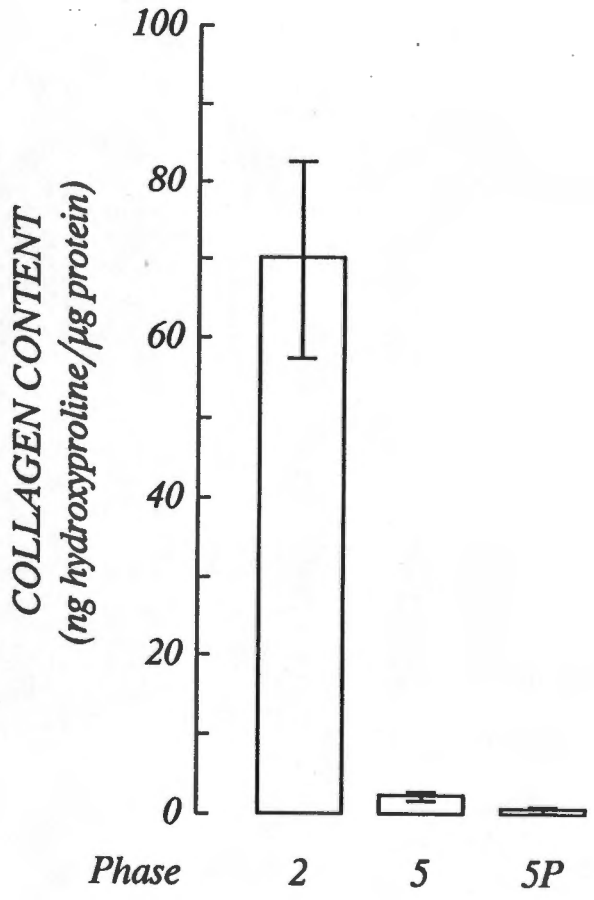


FIGURE 10

Collagen content of UCT-Mel 7 tumours at different phases of growth

The collagen present in UCT-Mel 7 tumours removed at different phases of growth was determined. The figure is constructed from the results of 6 phase 2 tumours, 4 phase 5 tumours and 12 phase 5P tumours.

The desmoplastic response was prominent only during phase 2.

FIGURE 11

A

B

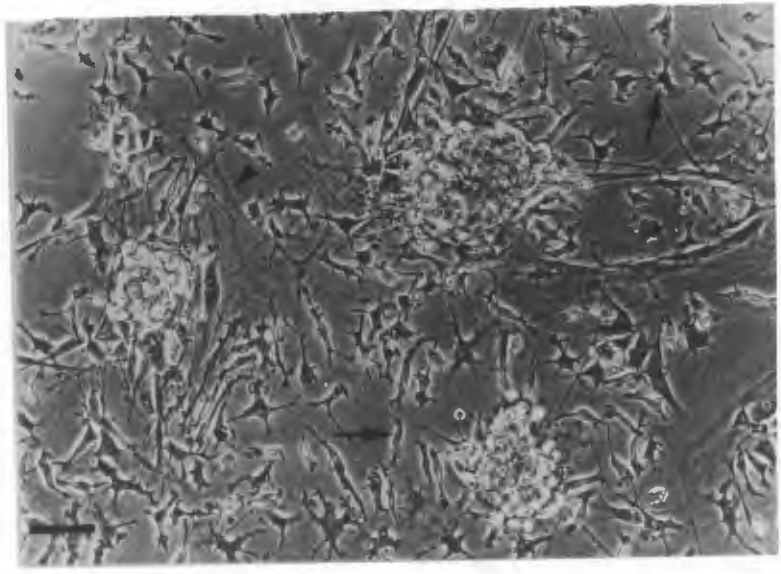


FIGURE 11

FIGURE 11

Cellular composition of UCT-Mel 7 tumours re-established in tissue culture

UCT-Mel 7 tumours were removed from mice and re-established in tissue culture. Host cell contamination varied between tumours. Scale marker = 50μm

Fig. 11a shows the cultures established from tumours removed during phase 5. A nest of tumour cells (arrow head) is shown with occasional macrophages (arrow) on a background of murine fibroblasts.

Fig. 11b shows the cultures established from tumours removed after two passages *in vivo*. Clusters of tumour cells are shown surrounded by occasional murine fibroblasts (arrowhead) and many murine macrophages (arrow).

FIGURE 12

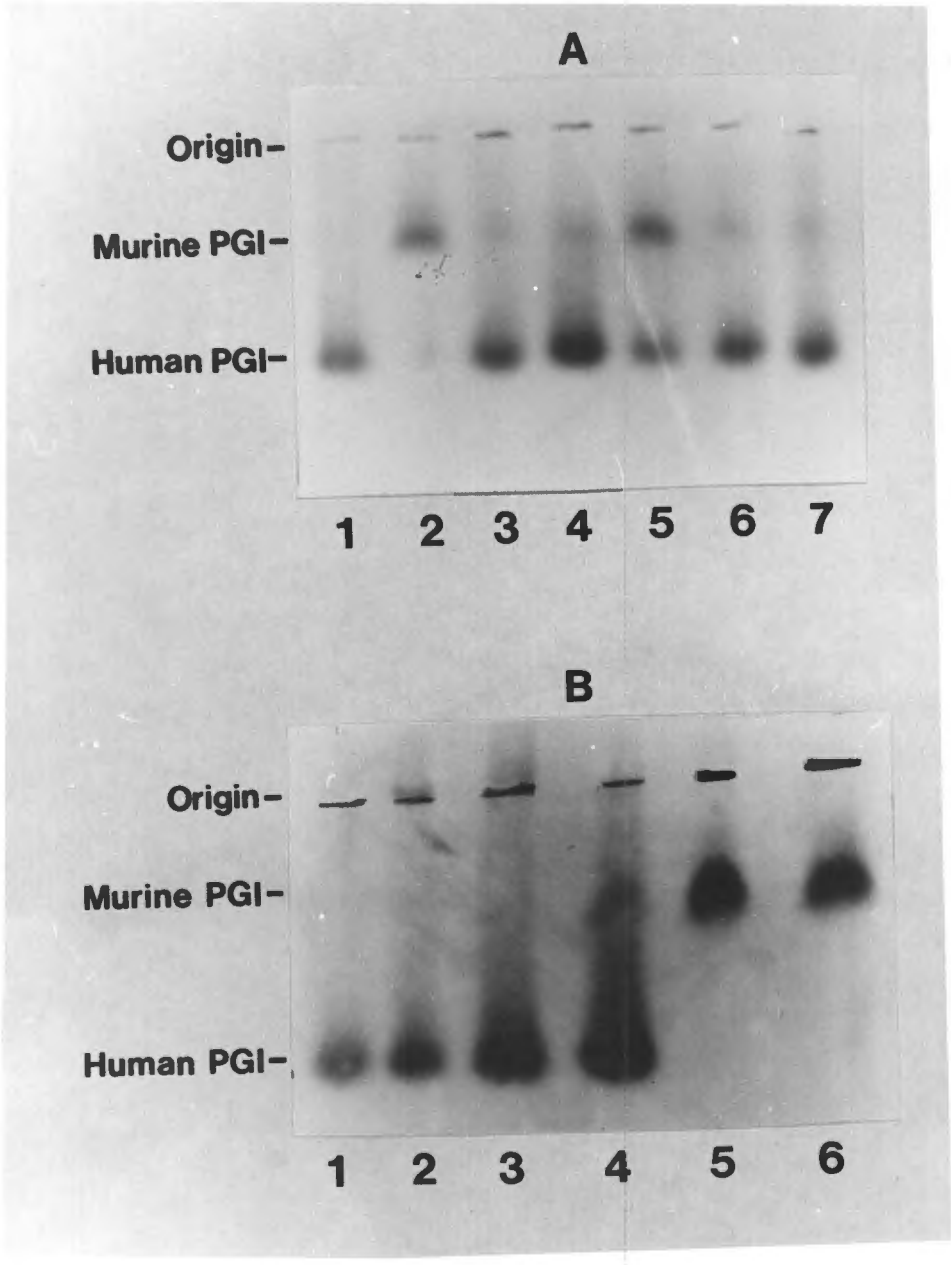

A
Origin-
Murine PGI-
Human PGI-
1 2 3 4 5 6 7
B
Origin-
Murine PGI-
Human PGI-
1 2 3 4 5 6

FIGURE 12

FIGURE 12

Relative contributions of human and murine cells to UCT-Mel 7 tumours grown in nude mice: phosphoglucoisomerase (PGI) zymography

Fig. 12a Tumours removed from the nude mice

Tumours were excised, homogenized and analysed electrophoretically as detailed in the Methods section, to determine the species and relative concentration of PGI enzymes present.
Homogenates were as follows:

Lane 1 - Human breast tissue showing a band with electrophoretic mobility of the human enzyme.

Lane 2 - Mouse liver homogenate, showing PGI enzyme band of mouse origin.

Lane 3 - UCT-Mel 7 tumour tissue homogenate, showing PGI bands of both human and mouse origin.

Lane 4 to 7 - UCT-Mel 7 tumour tissue homogenates from passage 1, 2, 2 and 3 respectively, showing both enzyme bands.

Fig. 12b Cells derived from tumours removed during phase 5P growth in vivo

As above, but in these samples homogenates were prepared from cells re-established as in vitro cultures from excised tumours.
Samples of cell lysates were as follows:

Lane 1 - Human breast homogenate, showing PGI enzyme band of human origin.

Lane 2 & 3 - UCT-Mel 7 cells from in vivo passage numbers 10 and 5 respectively showing exclusively human bands.

Lane 4 - UCT-Mel 7 cells from in vivo passage No. 7 showing a mixture of human and murine bands.

Lane 5 - UCT-Mel 7 cells from in vivo passage No. 5 showing only murine bands.

Lane 6 - Mouse liver homogenate, showing PGI bands of mouse origin.

In all analyses performed on lysates of tumours immediately after excision, I found a mixture of human and murine PGI's with the human isoenzymes as the predominant species. In interesting contrast, lysates examined after a period of re-established in vitro culture, occasionally contained predominantly murine PGI's. Of the 9 tumours re-established in vitro as pure cultures of tumour cells, 4 consisted of only murine cells despite the fact that they comprised a mixture of human and mouse cells at the time of excision with the human elements predominating. These murine cell lines were tumorigenic in syngeneic immunocompetent mice.

The six melanoma cell lines differed remarkably in the frequency with which they formed metastatic tumours (Table 2), varying from 6% in the case of UCT-Mel 1 to 100% with tumours of UCT-Mel 3. Tumours arising from UCT-Mel 7 cells consistently failed to give rise to metastases.

The chemotactic capacity of the UCT-Mel 7 cells was investigated using a standard micropore filter assay (Table 3). UCT-Mel 7 cells were shown to produce a soluble factor that was chemotactic for mouse peritoneal macrophages in a dose dependent manner. The amount of chemoattractant produced was dependent on the number of cells present.

The macrophages, UCT-Mel 7 cells and foreskin fibroblasts all reduced cytochrome c that was added to the medium. In doing so they displayed notable differences (Figs. 13 & 14, Table 4).

TABLE 2

Incidence of metastases

Cell line	No. of mice	Metastases		Maximum period of observation (days)	Site of metastases
		No.	%		
UCT-Mel 1	17	1	6	260	Lung
UCT-Mel 2	26	3	12	380	Lung
UCT-Mel 3	35	35	100	190	Lung, gonads, kidney, liver, intestine
UCT-Mel 4	10	1	10	270	Lung
UCT-Mel 5	22	3	14	313	Lung
UCT-Mel 7	10	0	0	370	-

The incidence of metastases was evaluated by detailed autopsy after excision of the primary tumour and a prolonged period of observation.

TABLE 3

Chemotactic activity of UCT-Mel 7

Chemoattractant	Mean Distance travelled (μm) ± SEM
0.5% Na caseinate	47.5 ± 3.7
Medium (RP10)	21.5 ± 4.5
UCT-Mel 7 conditioned medium	
1:2 dilution	51.3 ± 9.1
1:5 dilution	38.6 ± 6.2
UCT-Mel 7 cells	
$3x10^5$	68.1 ± 4.5
$0.6x10^5$	37.1 ± 5.7

The ability of UCT-Mel 7 cells to attract BALB/c peritoneal macrophages was assessed using a standard filter assay. Sartorius 8μm cellulose acetate depth filters separated two chambers; the upper one contained 10^6 macrophages/ml of medium and the lower contained either UCT-Mel 7 cells, UCT-Mel 7 cell-conditioned medium or appropriate controls.

Firstly, all of the cytochrome c reduction by macrophage products was superoxide dismutase (SOD)-sensitive indicating that the only reactive oxygen intermediate (ROI) released by these cells was superoxide anion (O_2^-). UCT-Mel 7 cells and foreskin fibroblasts, however, released reductants that were only partially sensitive to SOD (Fig. 13). The nature of the SOD-resistant ferricytochrome c reduction by these cells was not investigated further.

Secondly, O_2^- released by macrophages was highly inducible by PMA: addition of this compound at 10ng/ml caused an approximately 4-fold increase in the rate of ferricytochrome c reduction. Release of ROI's by fibroblasts and UCT-Mel 7 cells was constitutive and refractory to PMA induction.

Finally, in most experiments the release of O_2^- by PMA stimulated macrophages was linear for two hours after which it ceased. UCT-Mel 7 cells and fibroblasts released O_2^- linearly for three hours. SOD-resistant reduction of the ferricytochrome c by fibroblasts proceeded linearly for three hours whereas in the case of UCT-Mel 7 cells the rate diminished between 1 hr and 3 hrs after addition of ferricytochrome c to the medium.

In view of these results the rate of O_2^- released was calculated over the first hour and expressed as nanomoles of ferricytochrome c reduced per microgram protein per minute.

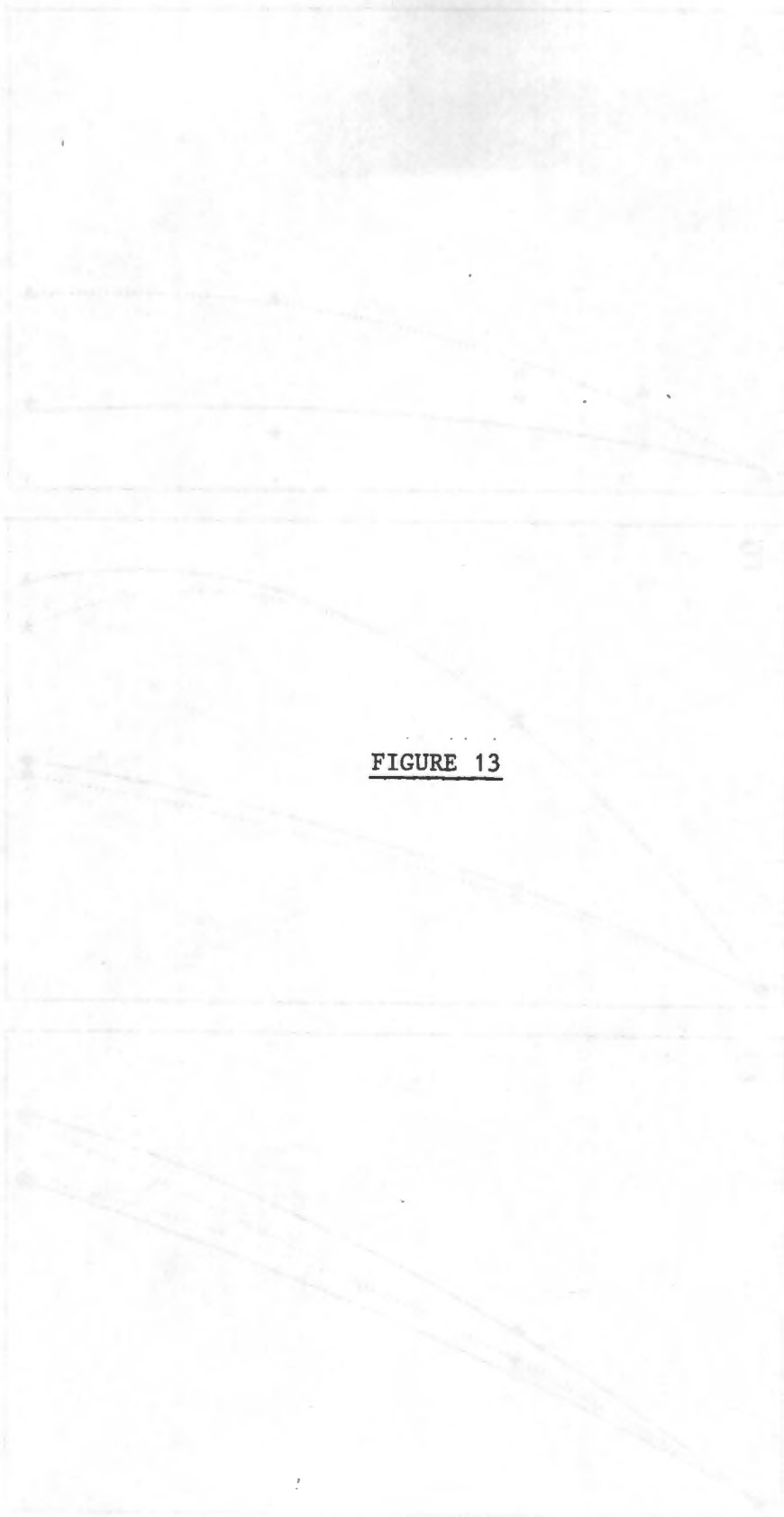


FIGURE 13

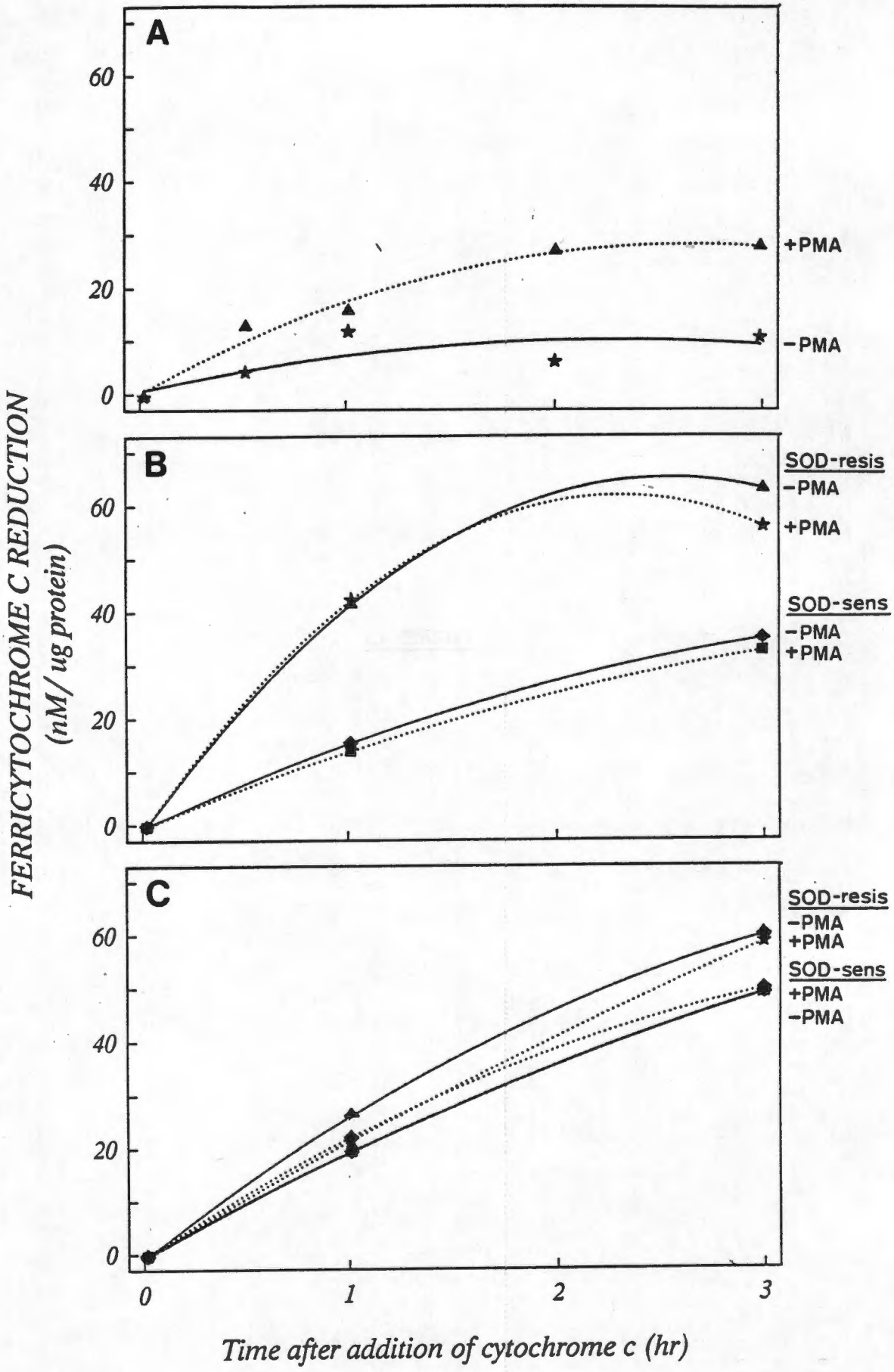

A
B
C
60
40
20
0
+PMA
−PMA
SOD-resis
−PMA
+PMA
SOD-sens
−PMA
+PMA
SOD-resis
−PMA
+PMA
SOD-sens
+PMA
−PMA
0
1
2
3
FERRICYTOCHROME C REDUCTION
(nM/ ug protein)
Time after addition of cytochrome c (hr)

FIGURE 13

FIGURE 13

Reduction of ferricytochrome c by cultured cells

The production of cytochrome c reductants by resting and PMA-stimulated (10ng/ml) mouse peritoneal cells, UCT-Mel 7 cells and foreskin fibroblasts was measured spectrophotometrically.

Murine macrophages (Fig 13a) only produced cytochrome c reductants when stimulated by PMA. All of the reductants produced were inhibitable by superoxide dismutase, indicating that the macrophages produced mainly superoxide anion in response to PMA.

UCT-Mel 7 cells (Fig. 13b) and foreskin fibroblasts (Fig. 13c) produced cytochrome c reductants constitutively. Not all of the reduction observed was inhibitable by superoxide dismutase indicating that these cells produced reductants, as yet unspecified, other than superoxide anion.

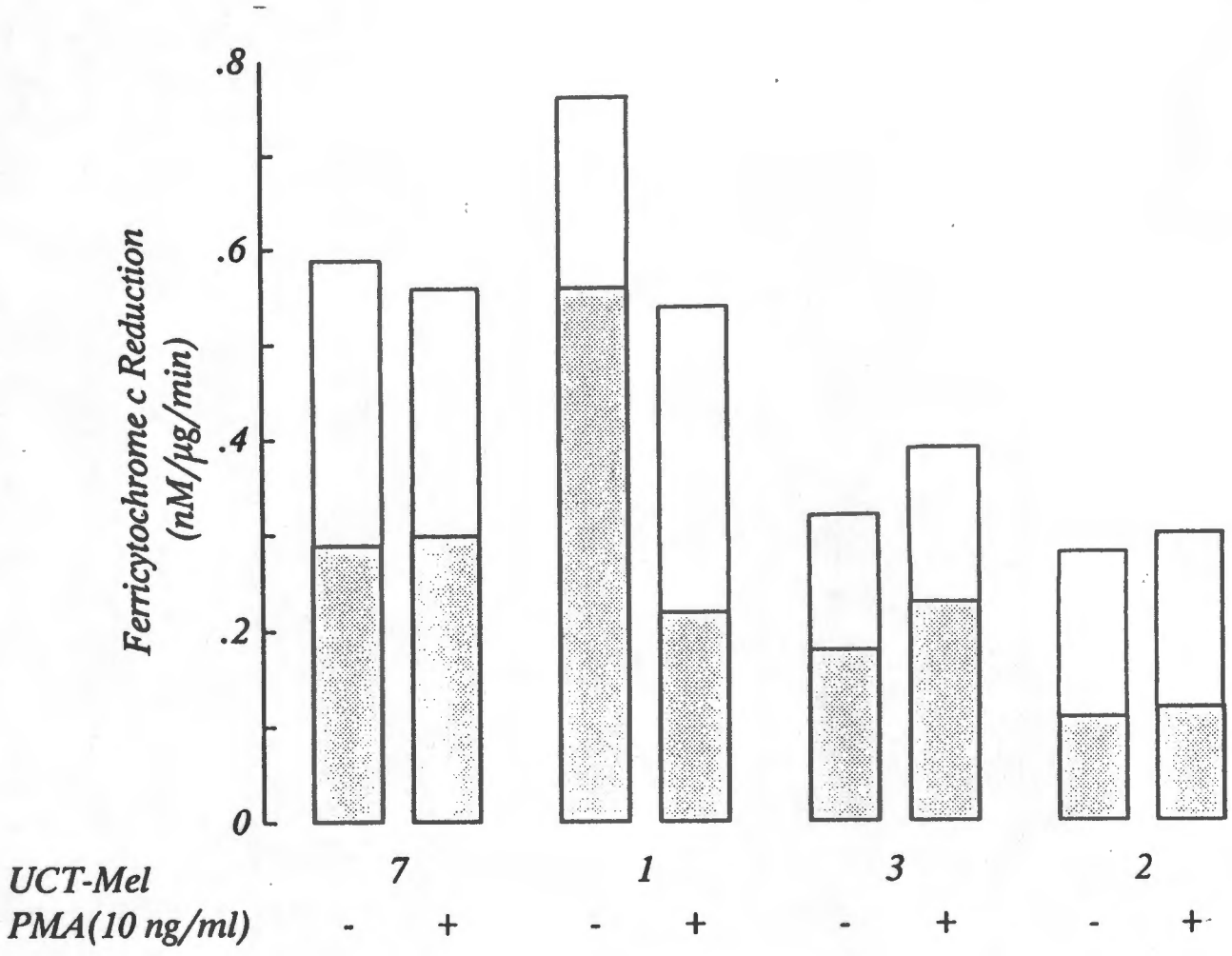


FIGURE 14

Ferricytochrome c reduction by different melanoma cell lines

SOD-resistant (hatched bars) and SOD-sensitive (open bars) reduction of ferricytochrome c by different melanoma cell cultures was measured with and without added PMA (10ng/ml).

Note that all cultures generated cytochrome c reductants, approximately half of which could be identified as superoxide anion.

Phorbol ester had no striking effect on the release of reductants and the rate of reductant release was unrelated to fibrogenic potential (cf. Fig. 8).

TABLE 4

Ferricytochrome c reduction: interaction between UCT-Mel 7 cells and peritoneal macrophages

Culture	Time (hr)	nM cytc red/μg protein/min			
		Expt 1		Expt 2	
		SOD-sens	SOD-resis	SOD-sens	SOD-resis
UCT-Mel 7 alone	1	0.37	0.17	0.35	0.24
	3	0.32	0.11	0.24	0.19
	7	0.19	0.08	0.10	0.10
M/phages alone	1	0.18	0	0.12	0
	3	0.11	0.03	0.11	0
	7	0.05	0.03	0.08	0.01
UCT-Mel 7 + M/phage observed	1	0.18	0.07	0.16	0.02
	3	0.09	0.08	0.10	0.05
	7	0.05	0.05	0.05	0.03
UCT-Mel 7 + M/phage expected	1	0.27	0.09	0.23	0.11
	3	0.21	0.07	0.17	0.09
	7	0.15	0.06	0.09	0.05

UCT-Mel 7 cells and macrophages were cultured, either alone or as co-cultures in medium containing 10^{-5}M ferricytochrome c. SOD-sensitive and resistant cytochrome c reduction was measured at 1, 3 and 7 hours after the addition of the pigment. Co-cultures generated less reductants than would have been expected from the results of the individual cultures.

Three other melanoma cell lines tested also released ferricytochrome c reductants, some of which were SOD-inhibitable and others not (Fig. 14). The reductants were released constitutively and production could not be enhanced by stimulation with PMA.

On two occasions, when UCT-Mel 7 cells and macrophages were co-cultured, the release of SOD-sensitive reductants was inhibited (Table 4). There was an approximate 30% reduction in the rate of ROI release observed over the first hour as compared to the expected rate.

DISCUSSION

The data I present in this chapter document the behaviour of tumours that developed when cells from a melanoma cell line, UCT-Mel 7, were inoculated into athymic mice. Compared to the other melanomas that I studied, these tumours were unusual in their phasic growth pattern; in that they elicited an intense desmoplastic response; and in the fact that, on occasions, they yielded malignant cells of murine origin after re-establishment in culture.

The phasic growth pattern was only seen after a prolonged period of repeated *in vitro* passage. Tumours that developed from 8th passage cells grew with the usual exponential kinetics whereas those from the 20th or subsequent passages grew to a plateau, regressed and subsequently burgeoned. These observations are readily explicable on the basis of *in vitro* selection for the "phasic phenotype".

Phenotypic drift of cell lines during the course of repeated in vitro passaging is well known (Neri and Nicolson, 1981; Welch et al., 1983) and is, in most cases, ascribed to selective forces that favour the emergence of subpopulations that have a proliferative advantage in the in vitro milieu. The differences that I observed in the doubling times of UCT-Mel 7 cells are consistent with this view. Cells at the 52nd passage had a doubling time (47 hours) that was approximately half that of the 8th passage cells (103 hours).

Continued in vitro passaging further selected for cells that produced tumours whose initial growth was so slow that the "plateau" and "regression" phases were not seen. These tumours (exemplified by those derived from 56th passage cells) eventually (i.e. after a period of approximately 6 months) became rapidly growing and lethal tumours. Here, too, selection for cells that proliferated rapidly in vitro could be documented (Fig. 7).

In many ways the growth pattern of the later passage cells showed the features of "dormancy" that are well described for many human tumours and for which few animal models exist. The tumour dormant state has been defined as "the state in which tumour cells persist in a host for prolonged periods of time with little net growth and which is terminated by an event which permits their rapid outgrowth" (Weinhold et al., 1979).

The first person to draw attention to the concept of the dormancy was Hadfield (1954) who recorded the long intervals of time that

frequently elapsed between excision of the primary tumour and the eventual reappearance of identical tumours at a much later stage. In clinical situations and in most experimental systems described (Eccles and Alexander, 1975; Gimbrone et al., 1972; Noble and Hoover, 1975; Weinhold et al., 1979) the local or metastatic tumours that terminate dormancy are, to all intents and purposes, the same as the primary tumour. It is, therefore, generally believed that residual cells remain after excision and that their growth is restrained by such factors as immunological suppression (Eccles and Alexander, 1975; Weinhold et al., 1979), lack of vascular supply (Gimbrone et al., 1972), hormonal deprivation (Noble and Hoover, 1975) or the concomitance of surgical "stress" (Fisher and Fisher, 1959). Phenotypic changes in the residual cells have not been emphasized as the "events" that terminate dormancy.

The experiments that I describe illustrate, quite clearly, that dormancy may be terminated by an alteration in the innate characteristics of the restrained cells. The lines that grew during stage 5 remained, when passaged into new recipients, rapidly growing neoplasms that showed none of the growth or other characteristics of the initial tumours.

I have no definitive explanation for the phenotypic changes that did occur, but several possibilities exist. The phase 5 tumours may have represented a reversion to the original rapidly growing phenotype that I observed when early passage cells were inoculated. Alternatively, they may have arisen as a result of genetic changes that conferred

growth factor independence or the capacity for the synthesis of other factors, such as angiogenic factor. In either event such developments would imply mutations in the slowly growing residual cell population - mutations that I felt may have been due to factors released by macrophages that infiltrated the tumours.

Tumours may produce chemoattractants for macrophages (Botazzi et al., 1985; Mantovani et al., 1986; Meltzer et al., 1977) and, in one case (Benomar et al., 1987), this could be correlated with the macrophage content of the tumours in vivo. I have shown that the UCT-Mel 7 cells are no exception in this respect (Table 3). Conditioned medium from cultures of UCT-Mel 7 cells were chemotactic for macrophages and significantly more so than similar harvest fluid from other melanoma cells that did not show the same degree of macrophage infiltration.

Phagocytic cells are also known to have mutagenic properties. Neutrophils have been shown to produce mutations in bacteria (Weitzman and Stossel, 1982) and in mammalian cells (Weitberg et al., 1983; Weitzman et al., 1985) and tumour-associated macrophages harvested from mouse mammary tumours increased the rate of mutation of the TA98 strain of Salmonella typhimurium (Fulton et al., 1984) and led to the emergence of drug-resistant variants in mouse mammary tumours at frequencies that were 5 to 10-fold higher than the spontaneous frequency (Yamashira et al., 1983).

Since phagocyte-induced mutagenesis is generally believed (Weitberg et al., 1983; Weitzman and Stossel, 1982; Weitzman et al., 1985) to be

mediated by active oxygen species that are generated by these cells (Johnston, 1978; Takemura and Werb, 1984), I studied the release of active oxygen metabolites by murine macrophages, fibroblasts, UCT-Mel 7 cells and other melanoma cells as measured by their ability to reduce cytochrome c.

I confirmed the fact that macrophages release superoxide anion as their principal or only cytochrome c reductants, but do so only when stimulated by agents such as PMA. If one assumes, in my experiments, that the tumour-associated macrophages were "activated", one might say that the presence of these cells in quiescent tumours was responsible for the eventual escape from dormancy. The matter, however, is far from resolved. If oxidative mutagenesis was primarily responsible for the change in phenotype of the residual cells, the importance of macrophages is put in doubt by the fact that the UCT-Mel 7 cells themselves released cytochrome c reductants, some of which were SOD-sensitive and others, of obscure nature, that were not. Furthermore, fibroblasts and other melanoma cells that did not show the growth kinetics of UCT-Mel 7 cells produced cytochrome c reductants at rates that were equivalent to those observed with UCT-Mel 7.

The case for the involvement of oxidative metabolism in the escape from tumour dormancy is further weakened by the demonstration of inhibition of release of ROI's in co-cultures of macrophages and melanoma cells. Other workers have documented the effect of malignant cell products on the release of reactive oxygen metabolites by macrophages (Szuro-Sudol and Nathan, 1982). My experiments have

extended these observations by showing that, in the case of melanomas at least, the reverse is also true: macrophages are able to inhibit the release of superoxide anion and SOD-resistant reductants by malignant cells.

Although the SOD-resistant reduction of ferricytochrome c by melanomas and fibroblasts was an interesting biochemical process and the interaction between macrophages and melanoma cells is potentially important, I decided, in the interests of pursuing the main objectives of this thesis, not to investigate them further for the time being. The experiments involving ROIs were initiated in the hope that they would shed light on the behaviour of this unique melanoma cell line in vivo in terms of its ability to induce a desmoplastic response and to show an unusual pattern of growth. Having satisfied myself that oxidative metabolism had little obvious to do with these aspects of this study, I set them aside to return to at some later stage.

In considering the role of macrophages as terminators of tumour dormancy, one should take into account mechanisms other than those that involve active oxygen species. It is well established, for example, that macrophages may promote tumour growth by releasing growth factors (Evans, 1977; Nelson et al., 1981), by stimulating angiogenesis (Polverini et al., 1977), by contributing to the establishment of a supporting stroma (Wahl, 1984), or by remodelling of the surrounding tissues in such a way as to favour invasion or spread (Henry et al., 1983).

There are currently two diametrically opposed theories as to the role of the macrophage in neoplasia. The original hypothesis, as put forward by Burnet in 1970 (Burnet, 1970), proposes the concept of "immune surveillance" in which macrophages, in conjunction with lymphocytes, monitor all tissues and eliminate any rogue cells before they can establish themselves. Although attractive in theory, this hypothesis has been difficult to validate experimentally. More recently, Prehn (1977, 1982) has put forward an "immunostimulatory theory" in which macrophages and immunocompetent cells, in fact, support the growth of the tumour, particularly during a "lymphodependent" phase early in progression.

A far more controversial theory has recently been proposed by Munzarova and Kovarik (1987) who suggest that somatic fusion takes place between macrophages and other cells, including neoplastic cells. This is followed by processes which culminate in malignant transformation of the hybrid cells or their progeny.

There have not been many reports on the functional status of tumour-associated macrophages except with regard to their cytotoxic potential (Evans and Lawler, 1980; Mantovani, 1978; Talmadge et al., 1981). Some workers have shown a correlation between the cytotoxic potential of tumour-associated macrophages and the immunogenicity and metastatic capacity of the tumour (Mantovani, 1978; Eccles, 1974). More recent work, however, failed to show any correlation between macrophage content and immunogenicity, metastatic capacity or <u>in vitro</u>

and *in vivo* growth rates of the tumour (Evans and Lawler, 1980; Evans and Eidlen, 1981; Talmadge et al., 1981). There is some indication that, in some instances, the functional capacity of the tumour-associated macrophages may be suppressed. My results and the results of others (Szuro-Sudol, 1982) attest to the capacity of certain tumours to inhibit the oxidative metabolism of macrophages. The ability of macrophages to respond to a chemoattractant may also be inhibited (Kalish and Brody, 1983; Snyderman and Pike, 1976).

It is of interest that, on four occasions in the experiments that I completed, neoplastic murine cells were isolated from cultures established from phase 5 tumours that comprised, at the time of their removal, malignant cells that were largely of human origin. The induction of malignant change in the stroma of transplanted murine tumours is a well-described phenomenon (Goldenberg and Pavia, 1981; 1982) and Giovanella and Fogh (1985) have recently drawn attention to the value of the xenografted nude mouse as a model for identifying the cellular origins of such tumours. Karyotypic analyses or isoenzyme electrophoresis of tumour tissues provided unequivocal identification of murine sarcomas that have developed at the site of human tumour xenografts in a number of reported instances (Goldenberg and Pavia, 1981; 1982). My observations contribute additional examples of this phenomenon.

Goldenberg and Pavia (1981, 1982), in reporting the appearance of malignant murine cells in xenografted human tumours stated that the phenomenon was only observed in human tumours first established in nude mice; tumours that resulted from the inoculation of established

human tumour cell lines did not induce stromal neoplasia. My results showed that their observations do not have general validity and that cell lines are, indeed, able to generate tumours that show "horizontal oncogenesis".

In certain cases (Goldenberg et al., 1971) the cells from recipient tumours contained both human and animal (hamster or mouse) genetic elements as definitive proof of the fact that cell fusion had occurred. This has led to the suggestion that "transforming genes" had been transferred to host stromal cells to cause malignant change. These suggestions draw support from the fact that murine cells that have been transfected with DNA extracted from human tumours do grow as transformed cells in culture and are tumorigenic in mice (Cooper, 1982; Shih and Weinberg, 1982; Weinberg, 1982). In most cases the transforming genes observed have proven to belong to the ras family (Der et al., 1982; Parada et al., 1982).

It is also possible, as I have suggested above, and as first proposed by Ehrlich and Apolant in 1905 (Ehrlich and Apolant, 1905) that the UCT-Mel 7 cells either alone or acting in concert with host inflammatory cells, generated an oncogenic microenvironment by the release of mutagenic substances.

Unfortunately I have no experimental data to contribute that might support either cell fusion or chemically induced mutagenesis as likely causes for cellular transformation. Tumour dormancy and horizontal oncogenesis are important features of neoplasia and it is my intention

to use the UCT-Mel 7:murine stromal cell system to examine this further with the modern genetic techniques that have recently become available.

Southern blots of the DNA extracted from UCT-Mel 7-induced host tumours probed with human Alu probes, as was done by Shih and Weinberg (1982), would be an obvious and initial experiment to perform to identify human sequences and the possible human transforming genes in the ostensibly murine neoplasms that develop in vivo.

Desmoplasia, defined as "the production of fibrous connective tissue within and surrounding a tumour" (Liotta, 1982; Robbins et al., 1984) was a prominent feature of UCT-Mel 7 derived tumours as shown by the prominent presence of collagen and reticulin in the histological specimens; the high content of hydroxyproline in tumour hydrolysates; and the large numbers of murine stromal cells present in these tumours.

Despite the prominence of stromal elements in so many human tumours, remarkably little effort has been made to assess its significance or to devise experimental models that could be used to elucidate its genesis. Seemayer et al. (1980) and Schurch et al. (1981) in recording the association of myofibroblasts with various neoplasms, suggested that proliferation of these stromal cells forms part of the host response to neoplasms - particularly neoplasms that are weakly antigenic and that grow slowly - and that this leads to containment of the tumour so that invasion and metastatic spread occur less readily (Schurch et al., 1981). It is interesting, in this regard, to note that of all the melanoma cell lines that I studied, only UCT-Mel 7

cells gave rise to desmoplastic tumours and these were the only tumours that did not metastasize.

The implicit conclusion to be drawn from these correlations is that a desmoplastic response is beneficial in the sense that it "curtails" dissemination of the tumour - a conclusion that is supported by the observations of others who have shown that the inhibition of the desmoplastic response to murine tumours by the administration of proline analogs (Barsky and Gopalakrishna, 1987) or corticosteroids (Takahashi and Biempica, 1985) increased the incidence of metastasis. On the other hand, as I have written previously (cf Introduction) clinical experience does not always attest to a beneficial relationship between desmoplasia and prognosis.

The induction of an intense stromal response by UCT-Mel 7 derived tumours constitutes the major concern of the remainder of this thesis and is taken up in the chapters that follow.

CHAPTER 2

THE EFFECTS OF MACROPHAGES ON COLLAGEN SYNTHESIS BY FIBROBLASTS

When malignant cells are transplanted - either as a single cell suspension or as a tissue fragment - into a subcutaneous site in an experimental host, their development into a viable tumour is critically dependent upon the establishment of a stroma. This loosely defined mesenchymal structure represents the supportive contribution of the host to tumorigenesis and comprises a complex mixture of interstitial collagens, glycoproteins, proteoglycans, basement membranes and blood vessels (Liotta, 1982).

Solid tumours have an obvious and absolute requirement for an adequate blood supply and it is thus not surprising that the angiogenic component of stromal function has been emphasized in research into host:tumour interactions. Developments over the past decade, however, have increasingly drawn attention to other ways in which the stroma exerts important effects upon cellular behaviour.

Stromal components are known, for example, to affect cellular morphology (Ben-Ze'ev et al., 1988; Blum et al., 1987; Hay, 1982; Montesano et al., 1984; Sugrue and Hay, 1981), differentiation (Chen and Bissell, 1987; Fujita et al., 1987; Reh et al., 1987; Spray et

al., 1987), substrate adhesion and patterns of migration (Grabel and Watts, 1987; Le Douarin, 1984) when added to in vitro cultures of diverse cell types. The most striking of these stromal influences, however, are those that involve synergistic interactions between cells, extracellular matrix (ECM) and growth factors.

Experiments to determine the effects of ECM have usually involved plating cells on plastic surfaces that have been conditioned by prior treatment with isolated matrix components or an extract of basement membrane (Kleinman et al., 1987) or by co-cultivation of test cells with those, such as bone marrow stromal cells, that express matrix upon their plasma membranes (Gordon et al., 1987). Enzymes such as heparinase or chondroitinase, that catalyse the degradation of specific macromolecules, have been used to identify the components of complex matrices that are responsible for the effects obtained.

These approaches have been successfully used to show that ECM reduces both in amount and in numbers the need for growth factors to support optimum cell growth (Gatmaitan et al., 1983). ECM, by binding growth factors, is able to sequester them for subsequent release (Folkman et al., 1988; Vlodavsky et al., 1987), to focus and present them to responsive cells (Gordon et al., 1987; Roberts et al., 1988) and to protect them from proteolytic degradation (Saksela et al., 1988). At a higher interactive level, the presence of ECM may modify or mediate the cellular response to growth factors (Madri et al., 1988; Salomon et al., 1981).

TGF-β, for example, inhibits the growth of endothelial cells plated on uncoated plastic; when added to the same cells plated on a collagen gel, it induces a proliferative response with the generation of a fine capillary network (Madri et al., 1988). The inhibition of endothelial cell growth by TGF-β, moreover, may well be an indirect effect mediated by fibronectin - an ECM component whose synthesis is induced by the growth factor (Madri et al., 1988). In the case of haemopoietic stem cells and endothelial cells, compounds such as heparan sulphate have been identified as the ECM component that synergises with GM-CSF and bFGF.

In chapter 1 I recorded the observations I had made on the cellular constituents of xenografted UCT-Mel 7 tumours and drew attention to the intense infiltration of these tumours with murine macrophages.

Macrophages may influence the growth and behaviour of tumours in a number of ways (reviewed in Chapter 1). In this chapter I focus upon the possibility that macrophages exert an indirect effect upon tumours by modulation of extracellular matrix deposition and composition. UCT-Mel 7-derived tumours shows both macrophage infiltration and abundant ECM and thus provided the experimental and conceptual points of departure for these studies to proceed.

Several reports attest to the degradative effects of macrophages on components of ECM. These cells release hydrolytic enzymes such as elastase (Banda and Werb, 1981), collagenase (Werb and Gordon, 1975; Mainardi et al., 1980), and plasminogen activators (Unkeless et al.,

1974), all of which have obvious relevance to the turnover of ECM and its remodelling. The plasminogen activators may provide local proteolytic activity for the activation of latent cellular collagenases (Werb et al., 1977).

Macrophage products, such as prostaglandin E_2 (Clark and Greenberg, 1987; Wahl et al., 1977), IL-1 (Laato and Heino, 1988; Postlethwaite et al., 1983; Postlethwaite et al., 1988), TNF (Bertolini et al., 1986; Dayer et al., 1985) and IFN-α (Duncan and Berman, 1987; Jimenez et al., 1984) may diminish collagen accumulation by inhibiting collagen synthesis in neighbouring cells or by increasing local cellular release of collagenases. Macrophage-derived factors are also known to inhibit fibroblast proliferation (Calderon et al., 1974; Kenyon et al., 1983; Korn et al., 1980).

On the other hand, macrophages could, in theory, bring about an increase in ECM deposition. They are known, for example, to stimulate angiogenesis (Polverini et al., 1977), fibroblast proliferation (DeLustro et al., 1983; Glenn and Ross, 1981; Schmidt et al., 1982) or collagen synthesis (Hibbs et al., 1983; Jalkanen et al, 1979; Jalkanen and Penttinen, 1982; Matsushima et al., 1985) either by released soluble factors or by direct cellular contact (Hibbs et al., 1983). By increasing procoagulant activity in tumours (Guarini et al., 1984; Lorenzet et al., 1983; Rambaldi et al., 1986) macrophages might increase local fibrin deposition (Rickles and Edwards, 1983) - a feature which is known to be associated with a desmoplastic response (Dvorak et al., 1981; Dvorak, 1986).

In more general terms, both clinical and experimental evidence exists to suggest a role for local macrophages as modulators of the fibroplasia that is seen in interstitial pulmonary fibrosis (Bittterman et al., 1982; 1983), progressive systemic sclerosis (Duncan and Berman, 1987; Korn, 1983; Worrall et al., 1986), rheumatoid arthritis (Pulkki, 1986) and other inflammatory conditions (Korn, 1980; Wahl, 1984). Wound healing is delayed by experimental manipulations that deplete macrophages or impair their function (Leibovich and Ross, 1975).

It was for these reasons that I investigated macrophage:fibroblast interactions that might plausibly have been responsible for the desmoplasia that I observed in UCT-Mel 7 tumours.

MATERIALS AND METHODS

Cells

Primary cultures of human fibroblasts were established, using standard procedures (Freshney, 1987), from fresh foreskins transported to the laboratory in tissue culture medium containing 5% FCS.
The cultures were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FCS (DB-10) and passaged as necessary. For experiments I describe in this thesis, the fibroblasts were used between passage numbers 4 and 10.

Two sources of murine macrophages were used: those obtained by peritoneal lavage and those of the cell line, $P388D_1$.

Resident mouse peritoneal macrophages were collected from 6-8 week old female mice by lavage with 4-5 ml of DB-1. The cells were pelleted by centrifugation at 400g for 5 mins, washed once in serum-free DB and adjusted to $1x10^6$ peritoneal cells/ml of serum-free DB.

For one experiment "elicited" peritoneal macrophages were harvested from animals in which a sterile peritonitis had been induced by inoculation of thioglycollate or other compounds. Details are given in the Results section.

$P388D_1$ is a mouse tumour line originally isolated by Dawe and Potter (1957) from a methylcholanthrene-induced lymphoid neoplasm (P388) in a DBA/2 mouse. It exhibits certain macrophage-like characteristics such as phagocytosis; Fc and complement receptors; and cytotoxicity in an antibody-dependent, cell-mediated cytotoxic system. It lacks other attributes such as chemotactic responsiveness and the enzyme adenosine deaminase (Snyderman et al., 1977; Koren et al., 1975). I obtained the cells from Dr. Lutz Thilo in 1983. The cells were maintained in RPMI-10.

Mice

Mice of the following inbred strains were obtained from stocks maintained in the UCT Animal House.

C3H/He//UCT; BALB/c//UCT; DBA/2//UCT; CBA/Ca//UCT.
Doubly deficient athymic nude mice designated UCT:NIH(S)II-nu/nu; and UCT:NIH(S)II-nu/+(furry heterozygotes) were obtained from stocks originally developed by Hansen (1977) and supplied by Dr. Giovanello, of the Cancer Research Laboratory, St. Joseph's Hospital, Houston, Texas.

Co-culture of macrophages and fibroblasts

Foreskin fibroblasts were plated at $1x10^5$ cells/35mm tissue culture dish (Falcon 3001) in DB-10. When confluent (usually 2-3 days after plating) the culture medium was changed to serum-free DB containing 25μg/ml ascorbate (Merck 127) and the cells incubated for 24 hours. The next day macrophages, ($1x10^6$ peritoneal cells/35mm dish) harvested as described, were added to the fibroblast cultures in serum-free DB containing ascorbate. The cells were co-cultured for 24 hours.

Fresh ascorbate was then added and the cultures labelled for 16 hours with 10μCi/ml [^{3}H]-Proline (Amersham TRK 323). The medium was collected and assayed for incorporation of radioactivity into newly synthesized collagen as described below.

Effects of peritoneal cells on fibroblast proliferation were determined directly by counting viable fibroblasts released from the plastic surface by trypsinization. The relative size of murine peritoneal exudate cells and human fibroblasts were such that I had no difficulty distinguishing them in the Neubauer counting chamber.

Radioactive Collagen Synthesis

Incorporation of [^{3}H]-Proline into collagen was assayed by the method of Webster and Harvey (1979). Briefly: acetic acid was added to the medium to a final concentration of 0.5M. Pepsin (Sigma P-7012) was then added to give a final concentration of 0.5mg/ml and the samples incubated at 4^{o}C overnight with shaking. Neutral salt-soluble rat skin collagen (Gross, 1958) was added to give a final concentration of 166μg/ml. The radioactive and carrier collagen was precipitated, first at an acid pH and then at a neutral pH. The final precipitate was washed once with 20% ethanol and then dissolved in 0.5M acetic acid for liquid scintillation counting. The results are expressed as dpm ^{3}H-incorporated/10^5 cells.

Recovery of labelled collagen internal standards exceeded 90% and more than 90% of synthesized salt-precipitable radioactivity was collagenase sensitive. Incorporation of [^{3}H]-Proline into collagen was linear with time over 24 hours (Fig. 1) and only about 1% of the total radioactive collagen in the dish was measured as intracellular collagen or deposited as a matrix adherent to the plastic surface; the remaining approximately 99% was found in the medium. Collagen synthesis accounted for approximately 44% of proline incorporated into

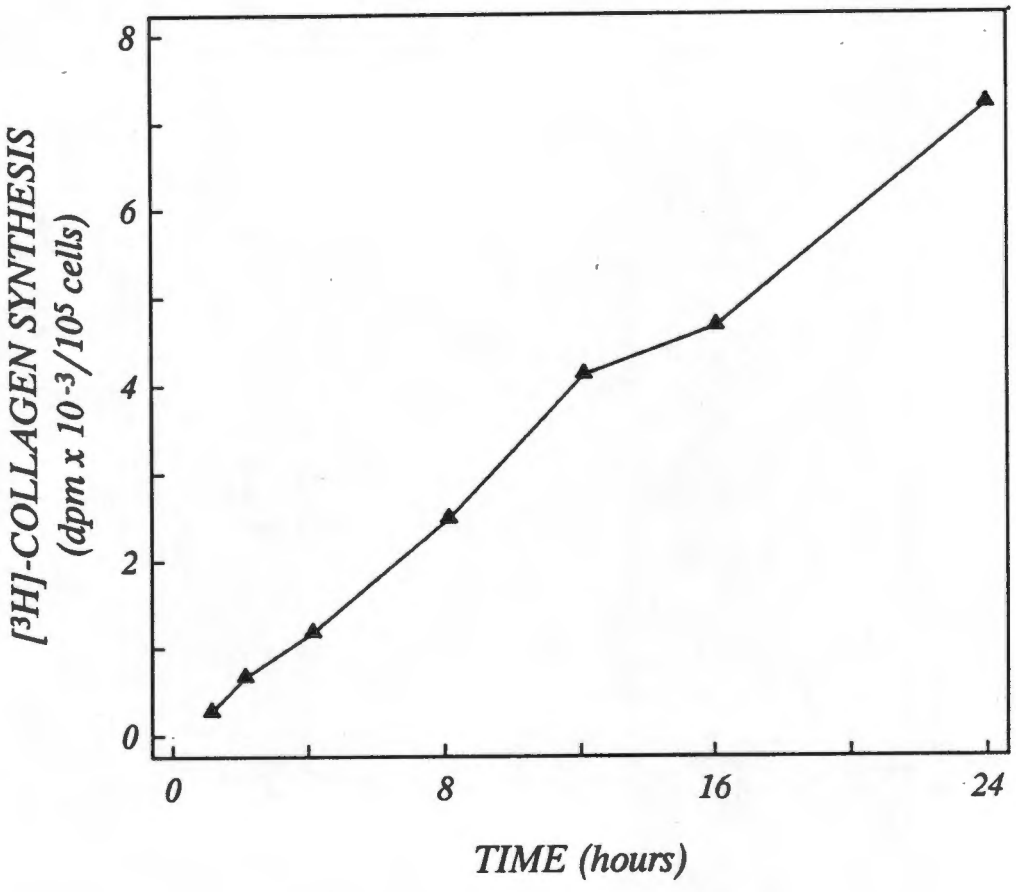


FIGURE 1

Incorporation of [^{3}H]-Proline into collagen

[^{3}H]-Proline (5μCi; 5μl) was added to confluent human foreskin fibroblast monolayer cultures (35mm dishes; ± 5x10^5 cells; 1 ml serum-free RPMI supplemented with 25μg/ml ascorbate) at time 0 to provide a medium proline concentration of 0.17mM and a specific activity of 63.25x10^6 dpm/μmole. The incorporation of [^{3}H]-Proline into collagen was measured, at the time points indicated, as described in the methods section.

Each point represents the average of 3 dishes.

The incorporation of [^{3}H]-Proline into collagen was linear with time over 24 hours.

TABLE 1

Collagen synthesis as a fraction of total protein synthesis

Labelled substrate	Incorporation of radioactive precursor into: Collagen (dpm)	TCA-precipitable protein (dpm)
[^{3}H]-Proline	12 309	27 815
[U-^{14}C]-Protein hydrolysate	722	6 143

Confluent foreskin fibroblast monolayers were covered with 1ml RP-10 supplemented with 25μg/ml ascorbate and containing 10μCi [^{3}H]-Proline or 10μCi uniformly labelled [U-^{14}C]-Protein hydrolysate (Amersham CFB.25). After 16 hrs incubation, the incorporation of [^{3}H] and [^{14}C] into collagen and TCA-precipitable protein was measured.

protein and approximately 11% of a mixture of uniformly labelled [^{14}C]-amino acids incorporated into TCA-precipitable material (Table 1).

The collagen produced was predominantly Type I with a small amount of Type III (Fig. 2).

The amount of collagen produced per cell was dependent on the number of fibroblasts on the dish at the time of labelling. After reaching a peak as the fibroblasts reached confluency, cellular collagen decreased with increasing cell density (Fig. 3). The negative regression and correlation coefficient were significant at a level intermediate between p=0.001 and p=0.01.

Collagenase assay

Radioactive collagen substrate for the collagenase assay was prepared by adding 50μCi of [^{3}H]-Proline (S.A. 15-40Ci/mmole; Amersham Cat. No. TRK 323) to 10 ml of RPMI-10 supplemented with 25μg/ml of ascorbate covering a confluent culture of 5x10^{6} foreskin fibroblasts in a 10cm dish. The cells were incubated for 16 h after which rat-tail collagen (approximately 1 mg) was added to the conditioned medium. The collagen was precipitated as described above to yield trypsin-resistant collagenase-sensitive protein with a specific activity of approximately 150 000 dpm/μg.

Collagenase was measured by a minor modification of Peterkofsky and Diegelmann (1971) by adding 1 ml of enzyme solution to approximately

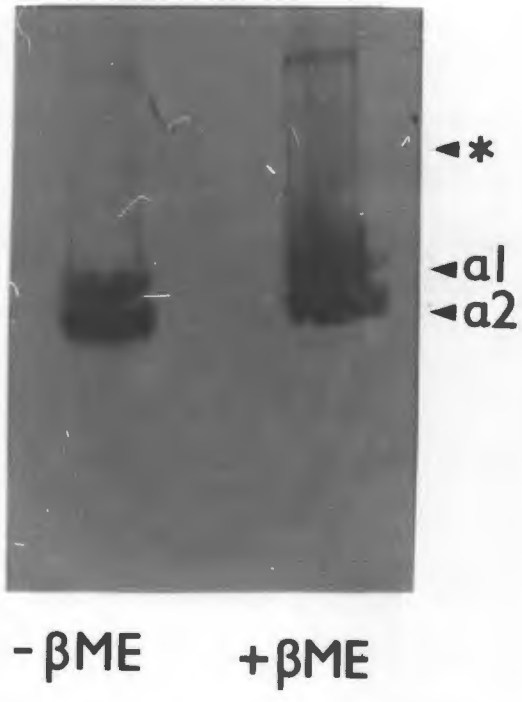


FIGURE 2

Type of collagen produced by human foreskin fibroblasts

Collagen synthesized by human foreskin fibroblast was labelled with 25μCi [^{3}H]-Proline for 16 hours. The labelled collagen was precipitated with ammonium sulphate, pepsin-treated, re-precipitated and then washed with 66% Ethanol. The final precipitate was dissolved in 0.1N NaOH and electrophoresed on a 7% sodium dodecyl sulphate polyacrylamide gel in the presence or absence of β-mercaptoethanol (βME). The gel was dried and autoradiographed using Kodak O-mat AR5 film.

The autoradiograph shows bands corresponding to α1(I) and α2(I) chains. The starred arrowhead marks the electrophoretic position of a band that was occasionally seen in reducing gels but not in non-reducing gels. This mobility and behaviour is typical of the α1(III) chain (cf Chapter 3 Fig. 2)

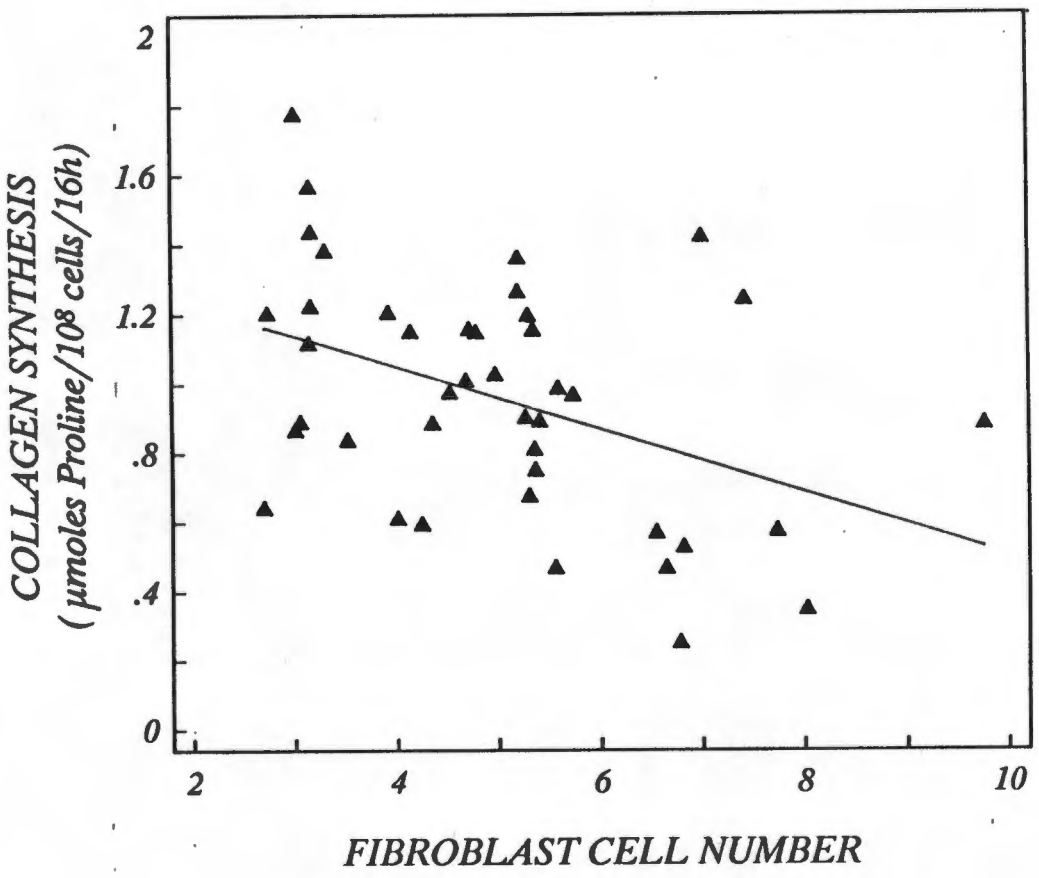


FIGURE 3

Inverse relationship between fibroblast cell number and cellular collagen synthesis

This figure depicts the rate of cellular collagen synthesis as a function of the number of fibroblasts on the dish. Each point is the average of 3 experimental dishes. The regression line was fitted by the method of least squares and had a correlation co-efficient of -0.425. The negative regression and correlation co-efficent were significant at a level intermediate between p=0.001 and p=0.01.

10 000dpm of radioactive collagen in 1 ml of 0.15M NaCl in 0.5M Tris HCl pH 7.5. The assay mixture was incubated at 37°C for 24h. Collagen hydrolysed was estimated as the difference in salt-precipitable radioactivity, after the addition of carrier collagen, before and after incubation.

Superinduction of $P388D_1$

$P388D_1$ cells were superinduced by a minor modification of the protocol of Mizel and Mizel (1981) in which cells were plated at $1x10^6$ cells/ml in DB-1. The following day 12-O-tetradecanoyl phorbol-13-acetate (PMA; Consolidated Midlands Corporation, New York, USA; final concentration of 10μg/ml) and cycloheximide (Sigma C-6255; final concentration of 10μg/ml) were added for 5 hours at 37°C. Actinomycin D (Sigma A-4262; final concentration of 1μg/ml) was added for the last hour. The cultures were then washed 4 times with DB and incubated for an additional 24 hours in DB-1. The superinduced conditioned medium was collected, centrifuged to remove cellular debris and stored at 4°C.

RESULTS

The addition of murine peritoneal cells to cultures of human foreskin fibroblasts had no effect on fibroblast proliferation but induced a two-fold increase in the rate of fibroblast collagen synthesis (Fig. 4). Medium that had been conditioned for 24 hours by incubation with the same number of peritoneal cells had no effect.

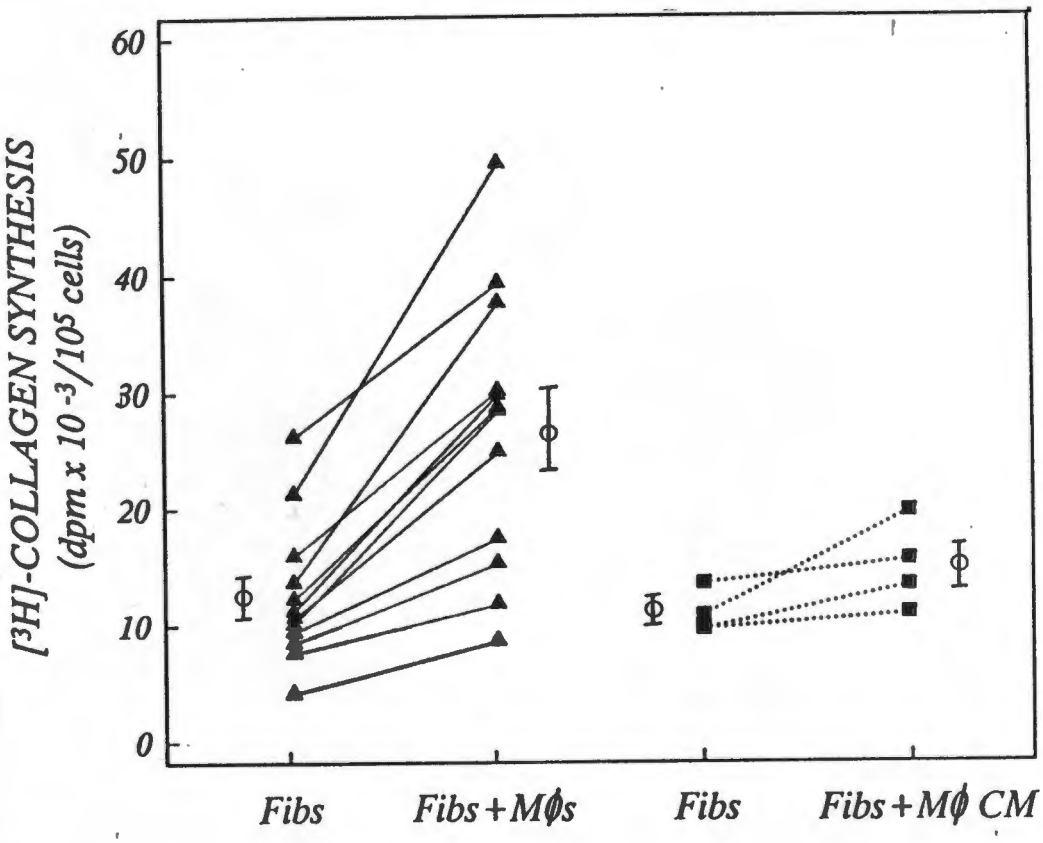


FIGURE 4

Effect of co-culturing with peritoneal macrophages on collagen synthesis by the fibroblasts

Confluent fibroblast monolayers (approximately $5x10^5$ cells/35mm dish) were serum-starved for 3 days with daily medium changes. The cultures were then covered with medium or medium that had been conditioned by $1x10^6$ peritoneal macrophages; or approximately $1x10^6$ peritoneal macrophages were added to each dish. After 24 hours each dish received 10μCi [^{3}H]-Proline. The cultures were processed for the measurement of [^{3}H]-Proline incorporation into collagen after 16 hours.

The graph depicts the experimental results represented as follows:

Each point represents the average rate of fibroblast collagen synthesis in triplicate cultures without (left) and with (right) added macrophages or macrophage conditioned medium. The average results obtained in each experiment are connected by a line. Open circles and vertical bars indicate means and standard errors, respectively. Student's t test for paired observations gave a value of t corresponding to $0.001 < p < 0.002$ for macrophages. Macrophage conditioned medium had no significant effect.

My failure to detect stimulatory activity in peritoneal cell-conditioned medium may have been due to a lack of activation of the peritoneal cells when these were incubated alone; co-cultivation may have stimulated them to release factors which then mediated the effect on fibroblasts. I accordingly collected supernatant medium from peritoneal cell:fibroblast co-cultures and added this to confluent fibroblasts. Slight stimulation of collagen synthesis was observed at high concentrations (1:2 or 1:5) only (Fig. 5).

Stimulation of collagen synthesis was directly related to the number of peritoneal cells added (Fig. 6); a maximal effect was seen at a peritoneal cell:fibroblast ratio of approximately 4:1.

Resident peritoneal cells and peritoneal cells that had been elicited by a variety of inflammatory agents stimulated collagen synthesis to a similar degree (Fig. 7).

Resident peritoneal cells from 6 different mouse strains stimulated collagen synthesis to an approximately equal extent and cells from athymic nude mice were as effective as those from heterozygotic litter mates or from other T cell sufficient strains (Fig. 8).

Graphic analysis (Eisenthal and Cornish-Bowden, 1974) of the kinetics of proline incorporation into collagen, indicated that the effect of adding peritoneal cells was to increase the maximal velocity of collagen synthesis from 1.3 μmoles proline incorporated/10^8 cells/16 hr to 4.4 μmoles proline/10^8 cells/16 hr. The apparent "Km" of the

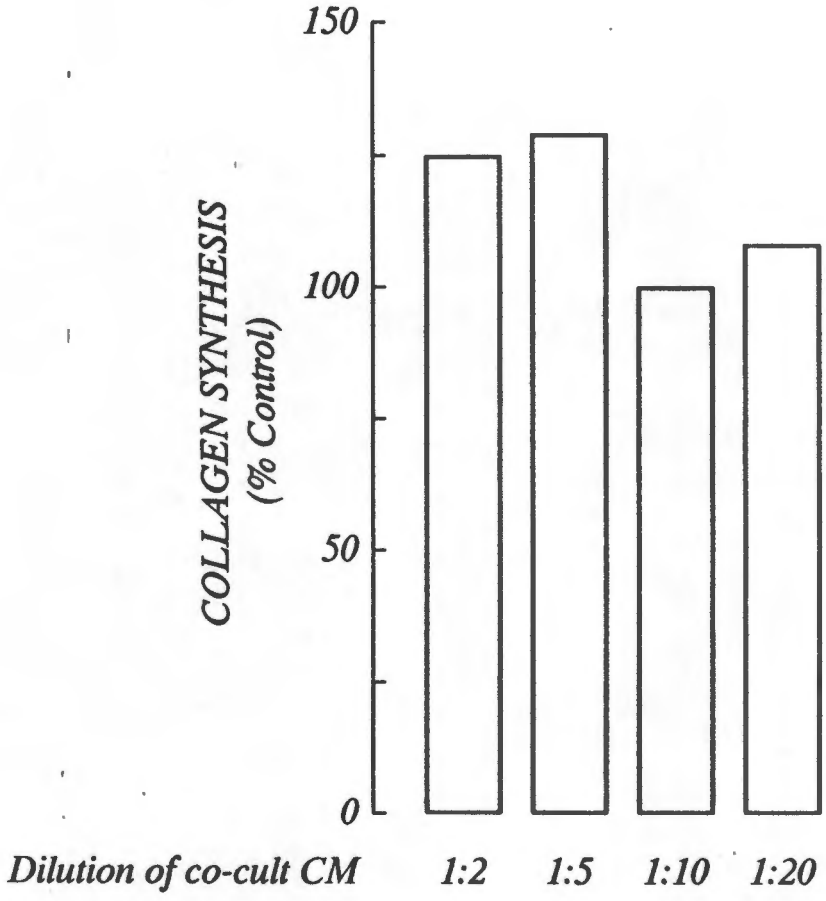


FIGURE 5

Effect of fibroblast:peritoneal macrophage co-culture supernatants on fibroblast collagen synthesis

Peritoneal cells ($1x10^6$/ml) and confluent fibroblasts were co-cultured in 10 ml of serum-free DB for 48 hrs. The conditioned medium was collected, spun and tested at the dilutions indicated for their abiliy to stimulate fibroblast collagen production. The results are expressed as a percentage of the control values obtained when supernatants of fibroblast cultures were added to the confluent fibroblasts.

At high concentrations (1:2 and 1:5) a slight stimulation of collagen production was observed.

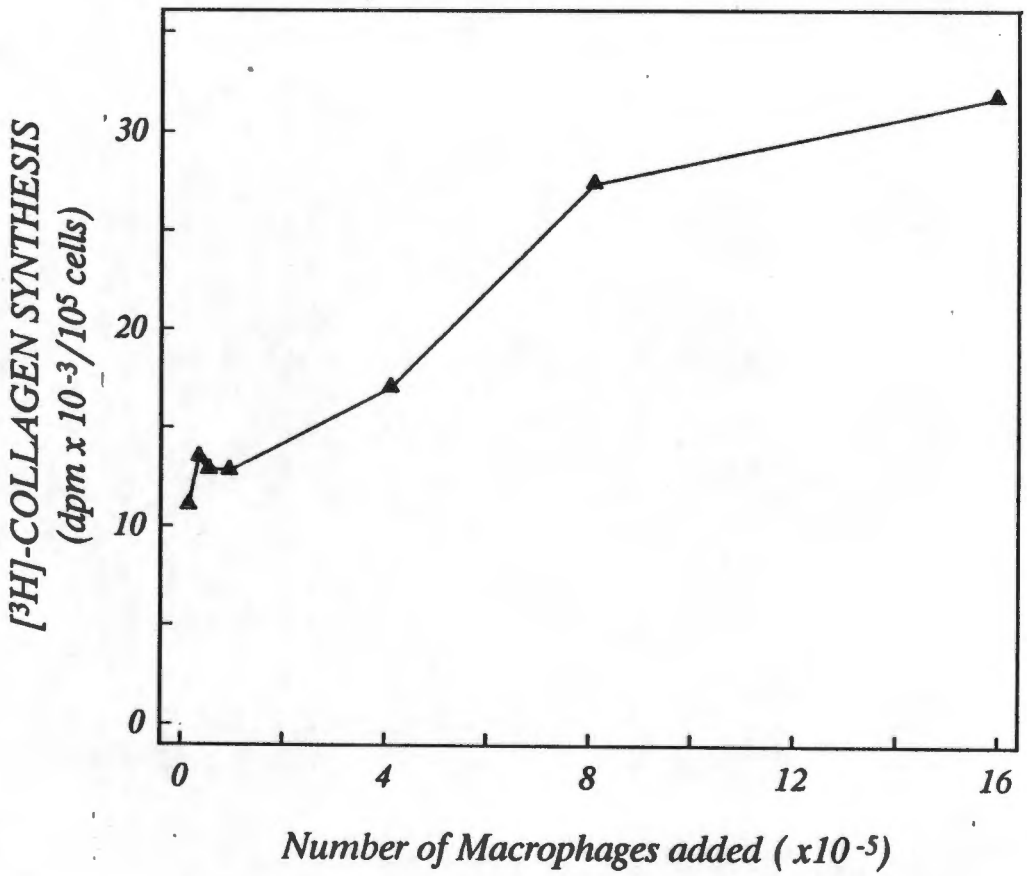


FIGURE 6

Dose-dependent stimulation of collagen synthesis by peritoneal macrophages

Confluent fibroblast monolayers were deprived of serum for 2 days, after which peritoneal cells at increasing concentrations were added to the fibroblasts.

After 24 hours, the cultures were labelled with 10μCi [^{3}H]-Proline. The cultures were processed for the measurement of [^{3}H]-Proline incorporation into collagen after 16 hours.

Increasing numbers of macrophages resulted in an increase in the stimulation of collagen synthesis by the fibroblasts.

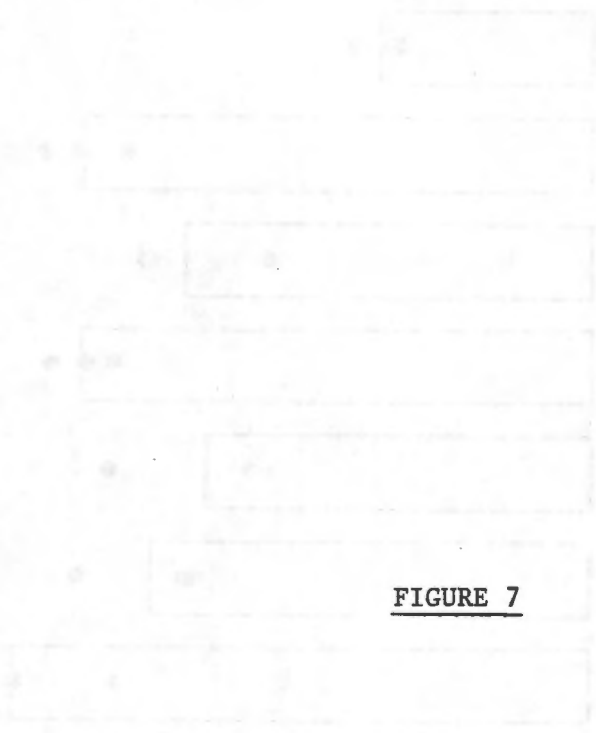


FIGURE 7

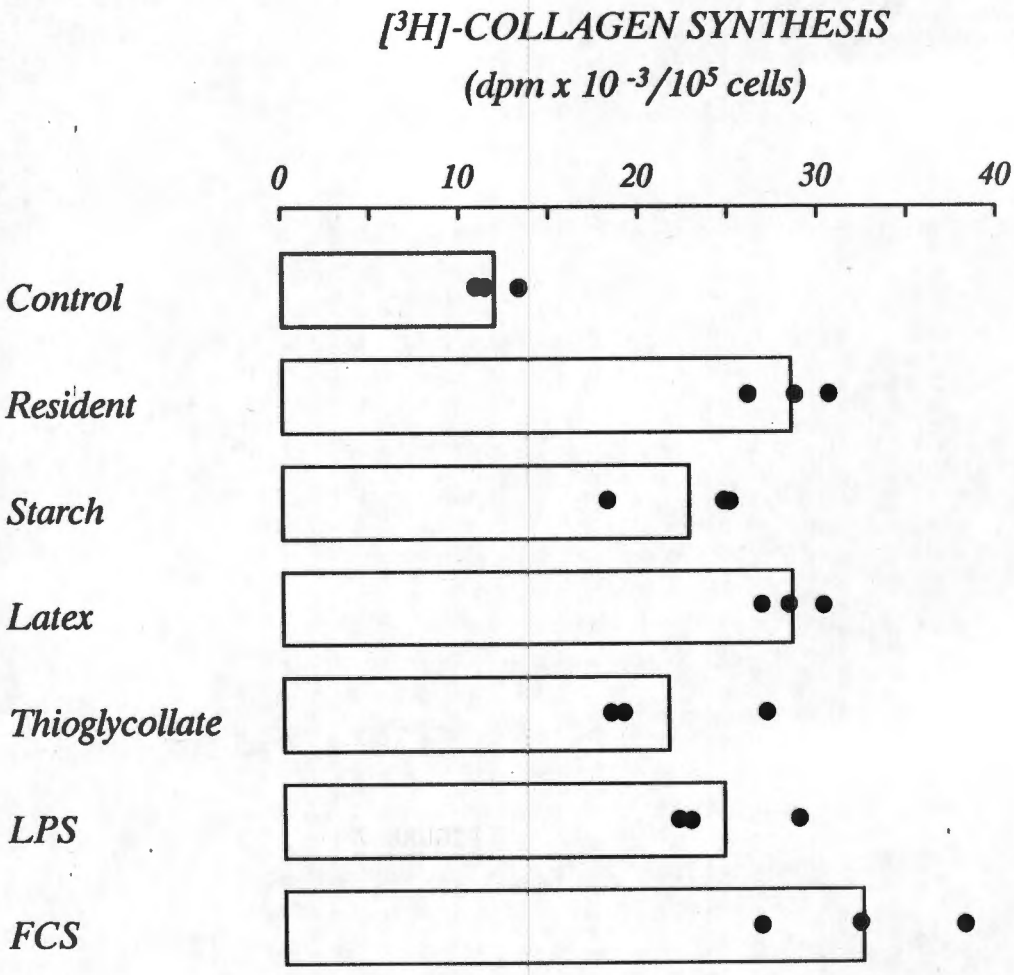

[3H]-COLLAGEN SYNTHESIS
(dpm x 10 -3/10^5 cells)
0
10
20
30
40
Control
Resident
Starch
Latex
Thioglycollate
LPS
FCS

FIGURE 7

FIGURE 7

Effect of the state of activation of peritoneal cells on their ability to stimulate collagen synthesis

Confluent fibroblast monolayers were deprived of serum for 1 day. Peritoneal cells, either resident or stimulated, were harvested from BALB/c mice and added to the fibroblasts at a concentration of $5x10^5$ cells/16mm well. The cells were co-cultured for 24 hours, labelled for 16 hours with 5μCi [^{3}H]-Proline, after which the cultures were processed for the incorporation of radioactivity into collagen.

Each point represents the average of the results of triplicate dishes for a single mouse. Bar heights represent the average of 3 mice.

Peritoneal cells were stimulated by intraperitoneal injections of:

1) Starch (2% suspension) or Latex (1:1000 dilution) 5 days before harvesting;
2) Thioglycollate (3% broth) or lipopolysaccharide (60 μg/ml) 4 days before harvesting;
3) Foetal calf serum, 1 day before harvesting.

Resident and activated cells stimulated collagen synthesis to a similar degree.

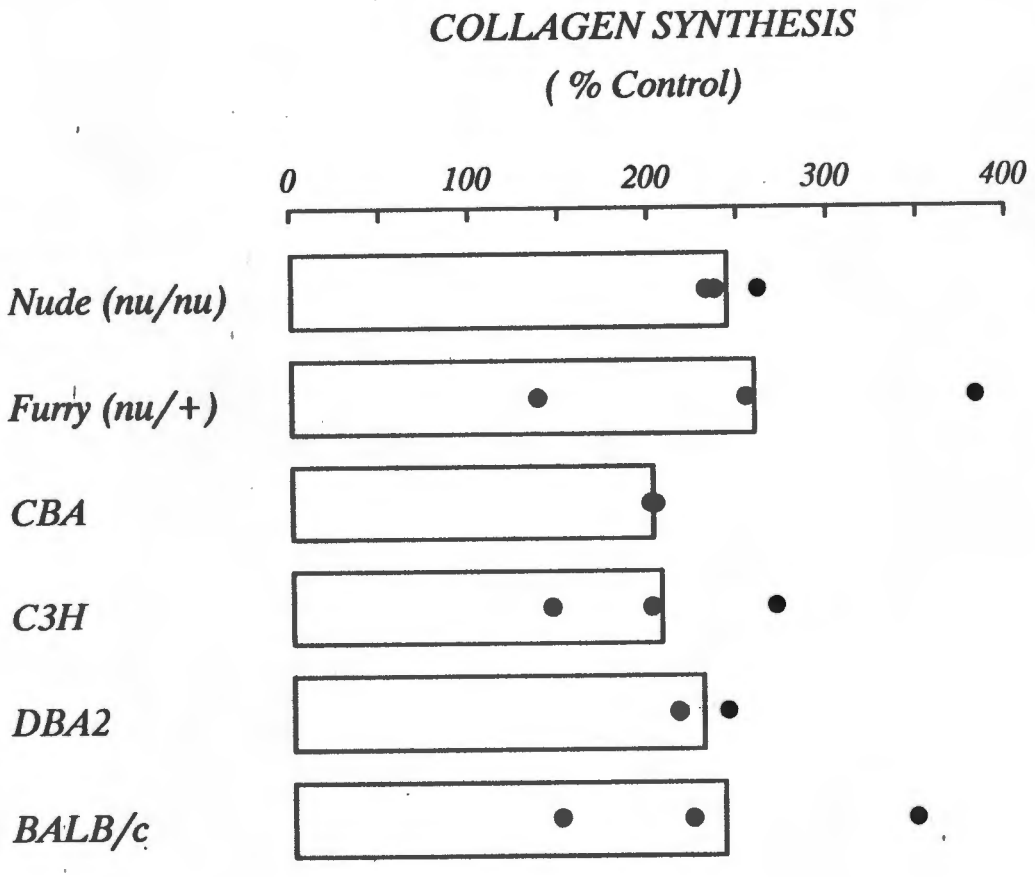


FIGURE 8

Comparative ability of peritoneal cells from different mouse strains to stimulate collagen synthesis

Confluent fibroblast monolayers were deprived of serum for 2 days. Resident peritoneal cells from the different mouse strains were added to the fibroblasts at $1x10^6$ cells/35mm plate. The cells were co-cultured for 24 hours and labelled for 16 hours with 10μCi $[^3H]$-Proline for the measurement of collagen synthesis.

The results are expressed as a % of the control values obtained when fibroblasts were cultured alone. Each point represents the mean value of the results obtained from 3 dishes in a single experiment. Bar heights indicate average values for all the experiments.

No significant differences were observed.

synthetic process (approximately 1.7mM proline) was unaffected (Fig. 9).

Lavage of the unstimulated peritoneal cavity of the mouse provides a limited number of cells of mixed composition with macrophages comprising only a variable proportion. The fibrogenic effects of soluble factors released by the macrophages present in such a cell population may thus have been quantitatively insignificant or obscured by the products of other cell types. I thus resorted to the use of macrophage-like cells of the $P388D_1$ line in the hope that they would provide a relatively pure cell population that could be induced to release factors in amounts that would offer better opportunities for characterization.

The addition of $P388D_1$ cells to fibroblast cultures had no appreciable effect on [^{3}H]-Proline incorporation but medium that had been conditioned by $P388D_1$ cells did cause a consistent, though slight and variable (23% - 86%) increase in collagen synthesis (Fig. 10).

Encouraged by these results, I added various compounds to the $P388D_1$ cultures in an attempt to induce them to release more of the factor(s) that stimulate fibroblasts to synthesize collagen. The effects of adding lipopolysaccharide to the $P388D_1$ cells were unremarkable in this respect. Induction with PMA or superinduction with PMA, cycloheximide and actinomycin D (Mizel and Mizel, 1981), however, caused the unexpected release of potent inhibitors of collagen synthesis. These results are summarized graphically in Fig. 10.

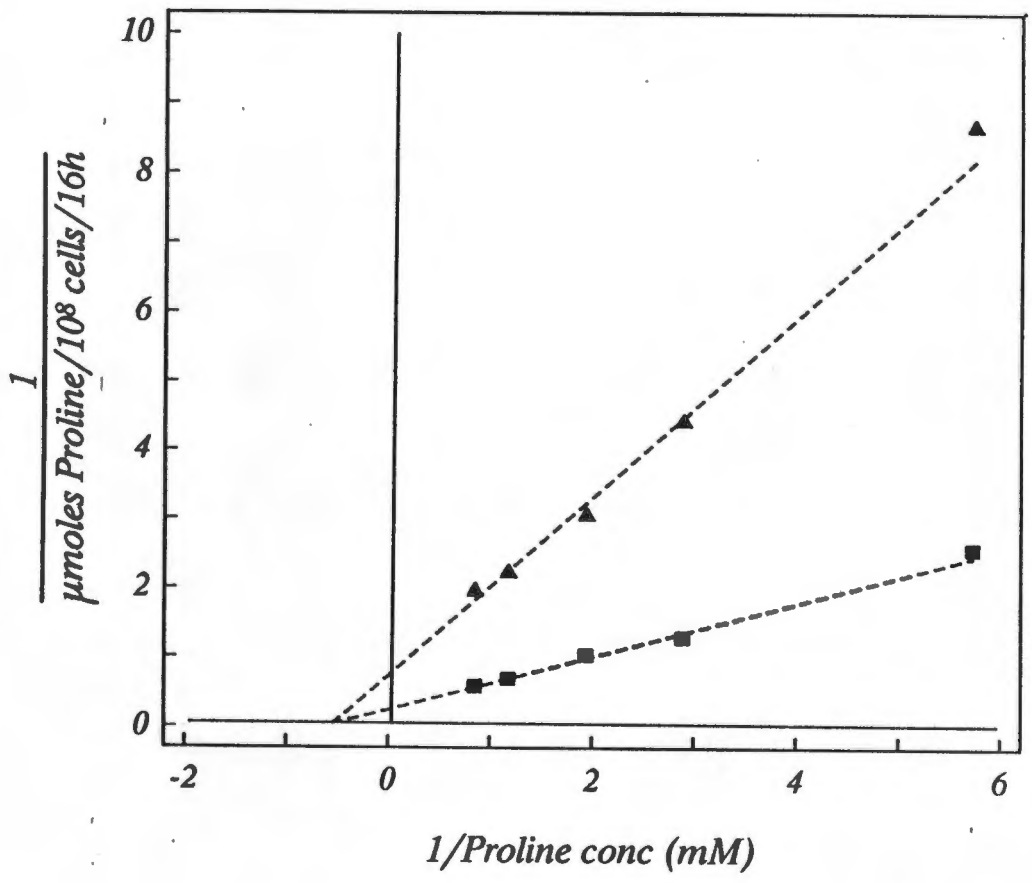


FIGURE 9

Lineweaver-Burk analysis of Proline incorporation

In this figure the reciprocal of the rate of proline incorporation into collagen (µmoles proline/10^8 cells/16 hr) has been plotted as a function of the reciprocal of the proline concentration (mM) for graphic analysis according to the method of Lineweaver and Burk.

Fibroblasts cultured in the presence of peritoneal macrophages ($8x10^5$/dish;■----■) and those cultured alone (▲----▲) showed similar apparent "Km" values (1.724mM) but different extrapolated maximal rates of incorporation (4.4 and 1.3 µmoles proline incorporated/10^8 cells/16 hrs, respectively).

FIGURE 10

FIGURE 10

Effect on fibroblast collagen synthesis of $P388D_1$ cells or conditioned media taken from these cells after stimulation

Confluent fibroblast monolayers were serum starved for 2 days. The cultures were then treated by adding $P388D_1$ cells or medium conditioned by $1x10^6$ of these cells cultured in 1 ml of DB after exposure to various compounds. Results are expressed as a percentage of the control values obtained from fibroblasts cultured alone. Each point represents the results of individual dishes; bar heights represent the average. The labels signify additions as follows:

Cells: $1x10^6$ $P388D_1$ cells added to each 35mm dish.

HF (1:5): Medium conditioned for 24 hours by $P388D_1$ cells, diluted 1:5 with fresh DB.

LPS HF: $P388D_1$ cells were stimulated with 20μg/ml lipopolysaccharide (LPS) for 48 hours and the conditioned medium added.

PMA HF: $P388D_1$ cells were stimulated with 10μg/ml PMA for 5 hrs. The cultures were then washed and conditioned medium was collected over the next 24 hrs.

Superinduced HF: $P388D_1$ cells were treated with PMA (10μg/ml), cycloheximide (10μg/ml) and actinomycin D (10μg/ml) for 5 hrs. The cultures were then washed and the medium conditioned over the next 24 hours collected.

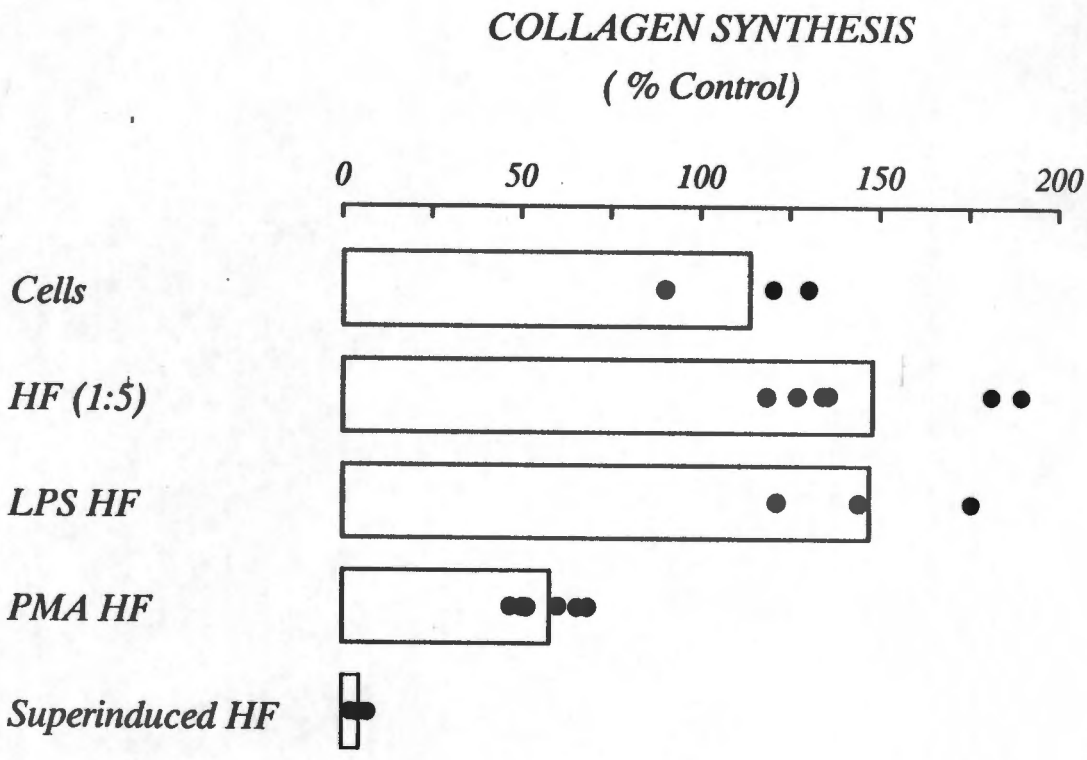

COLLAGEN SYNTHESIS
(% Control)
0
50
100
150
200
Cells
HF (1:5)
LPS HF
PMA HF
Superinduced HF

FIGURE 10

Although the cultures of P388D_1 cells were washed extensively after superinduction and before collection of the conditioned medium, it was none the less possible that the subsequent inhibition of fibroblast collagen synthesis could have been caused by carryover of trace amounts of PMA, cycloheximide or actinomycin D. I therefore titrated these compounds for their ability to inhibit collagen synthesis and found that 50% inhibitory concentrations of PMA, cycloheximide and actinomcyin D were 1.2ng/ml, 56ng/ml and 7.6ng/ml respectively. These concentrations were in excess of those that could have been present in harvest fluids from the washed superinduced P388D_1 cells.

The inhibitor showed the following characteristics: it was retained by a dialysis membrane; relatively heat stable and capable of withstanding 56°C for 30 minutes, 80°C for 10 minutes or 100°C for 1 minute without loss of activity; it was acid labile with loss of activity by exposure to pH 2.5 for 4h and very stable on storage, retaining its activity after 6 months storage at 4°C.

Inhibition of collagen synthesis by superinduced conditioned medium was dose dependent (Fig. 11) and reversible (Fig. 12). Although some batch variation was seen, in most cases, superinduced conditioned media inhibited fibroblast collagen synthesis by 50% at a dilution of about 1:300 and was toxic (by trypan blue exclusion) at a dilution of 1:5 or less.

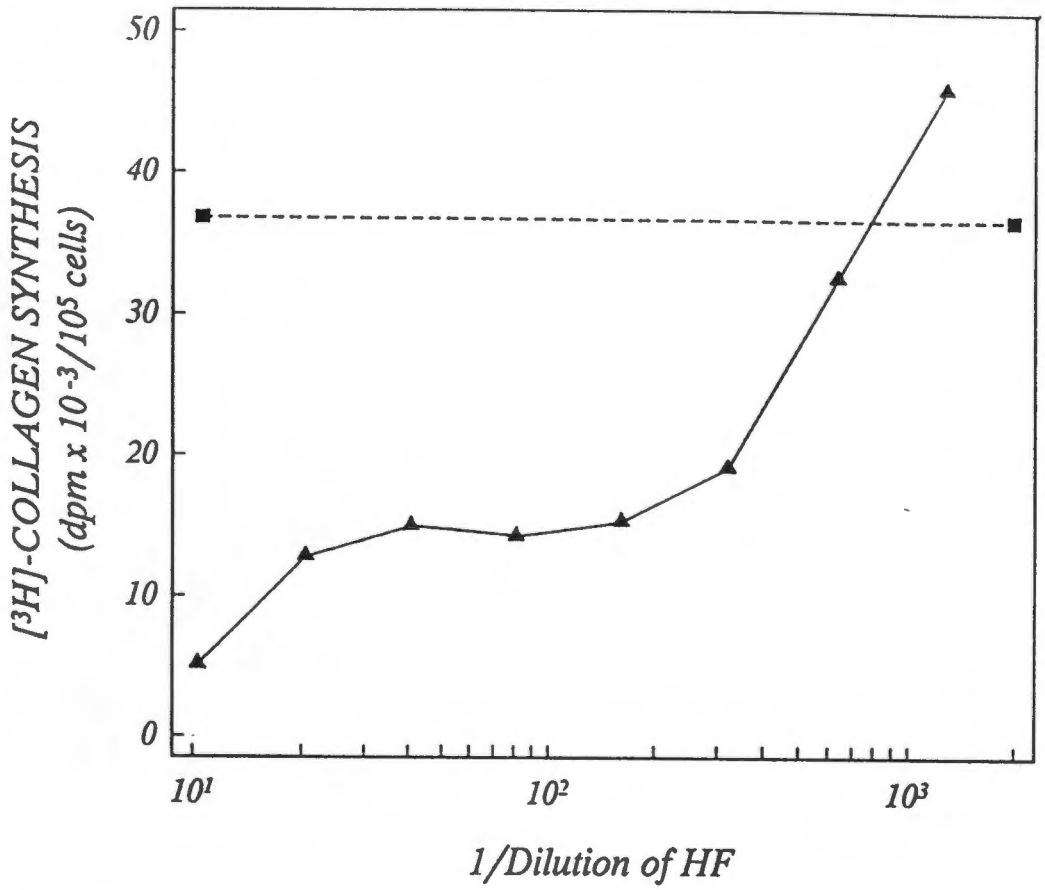


FIGURE 11

Dose-dependent inhibition of collagen synthesis by superinduced conditioned medium

Superinduced P388D_1 conditioned medium was prepared by treating the cells with PMA, cycloheximide and actinomycin D as described in the text and added to confluent fibroblast monolayers at the dilutions shown. After 24 hrs, the cultures were labelled for 16 hrs with 10μCi [^{3}H]-Proline for the measurement of collagen synthesis.

The rate of collagen synthesis in control cultures is indicated by the horizontal dashed line. Each point represents the average of the results of 3 dishes.

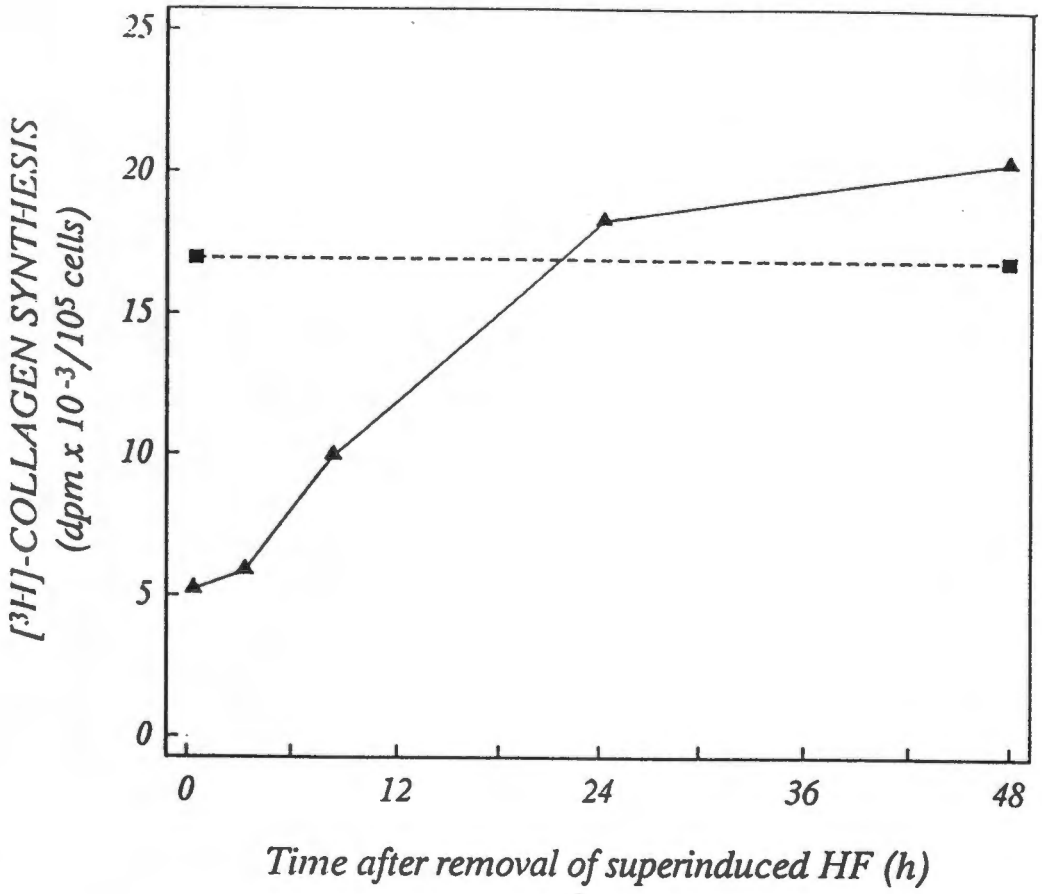


FIGURE 12

Reversibility of inhibition by superinduced conditioned medium

The superinduced conditioned medium, diluted 1:10 in DB-1, was added to confluent fibroblast monolayers for 48 hours. The cells were then washed and incubated for the indicated times before labelling with a 16 hr pulse of [^{3}H]-Proline. Radioactive collagen was then precipitated and counted as usual.

The rate of collagen synthesis in control cultures is indicated by the horizontal dashed line. Each point represents the average of the results of 3 dishes.

The effect of the superinduced conditioned medium is reversible. Collagen synthesis had reverted to control levels 24 hr after removal of the conditioned medium.

FIGURE 13

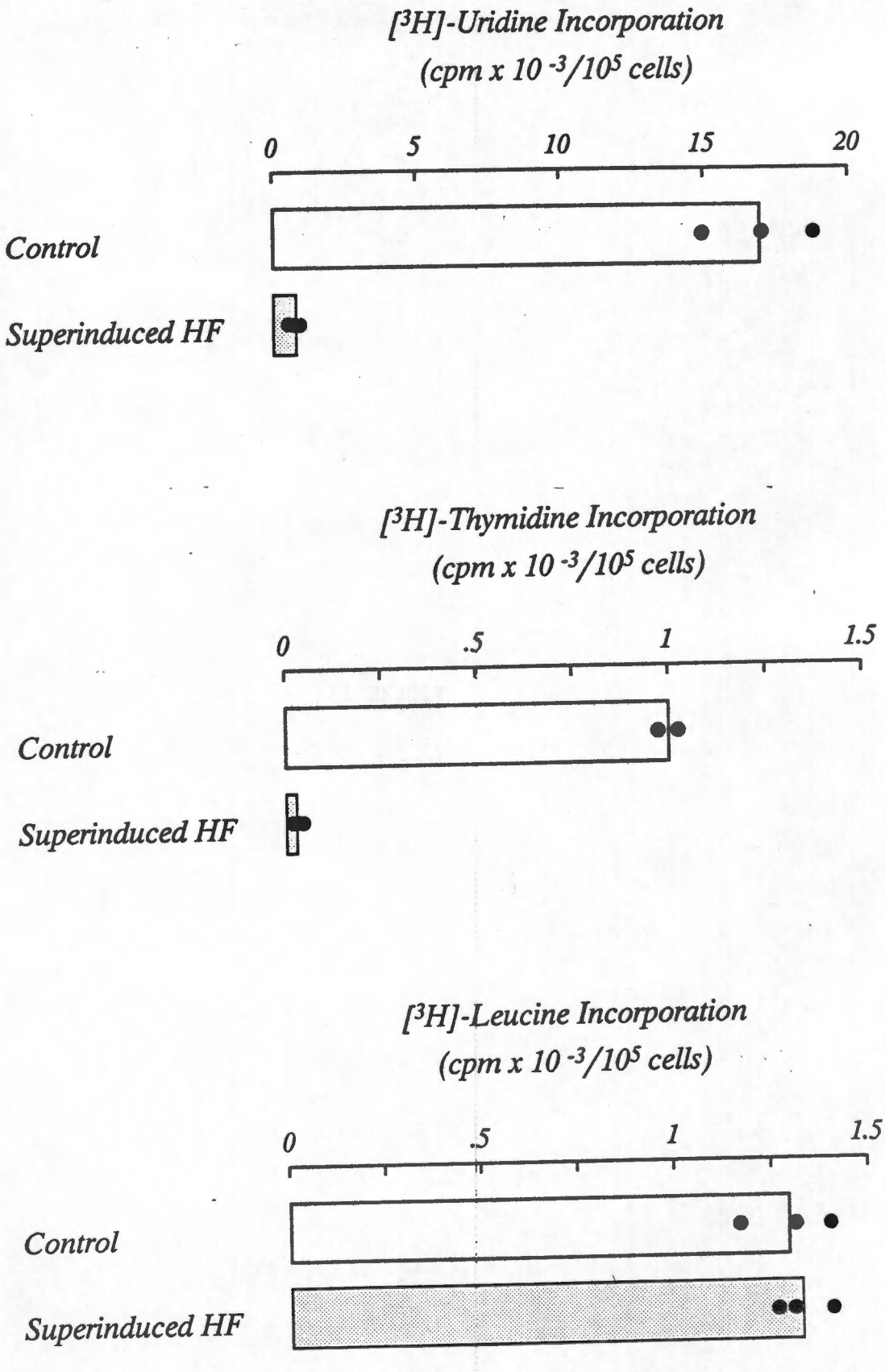

[3H]-Uridine Incorporation
(cpm x 10 -3/10^5 cells)
0
5
10
15
20
Control
Superinduced HF
[3H]-Thymidine Incorporation
(cpm x 10 -3/10^5 cells)
0
.5
1
1.5
Control
Superinduced HF
[3H]-Leucine Incorporation
(cpm x 10 -3/10^5 cells)
0
.5
1
1.5
Control
Superinduced HF

FIGURE 13

FIGURE 13

Effect of superinduced conditioned medium on macromolecular synthesis

Superinduced $P388D_1$ conditioned medium was diluted 1:2 in DB-1 and added to confluent fibroblast monolayers. After 24 hrs of incubation the cultures were pulsed for 5 hrs with [^{3}H]-Leucine,[^{3}H]-Uridine or [^{3}H]-Thymidine (5μCi in each case). The cultures were then washed with PBS and the labelled macromolecules precipitated with 5% TCA (final concentration) for 30 min at 4°C. Precipitates were collected and counted as described in the text.

Each point represents the results of individual dishes.
Bar heights represent the average results of 3 dishes for each set of experimental conditions.

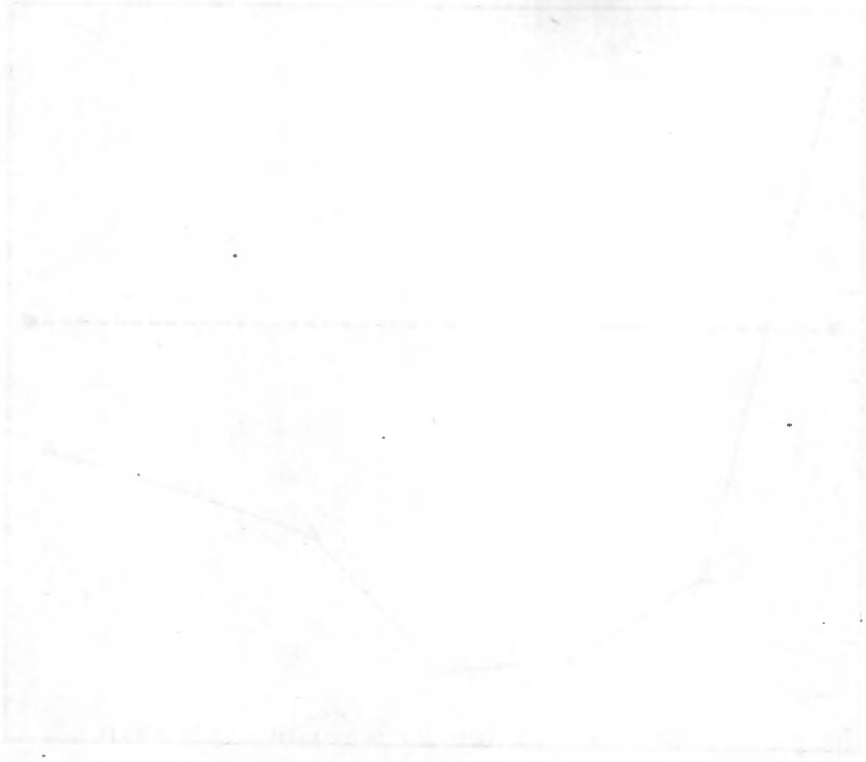


FIGURE 14

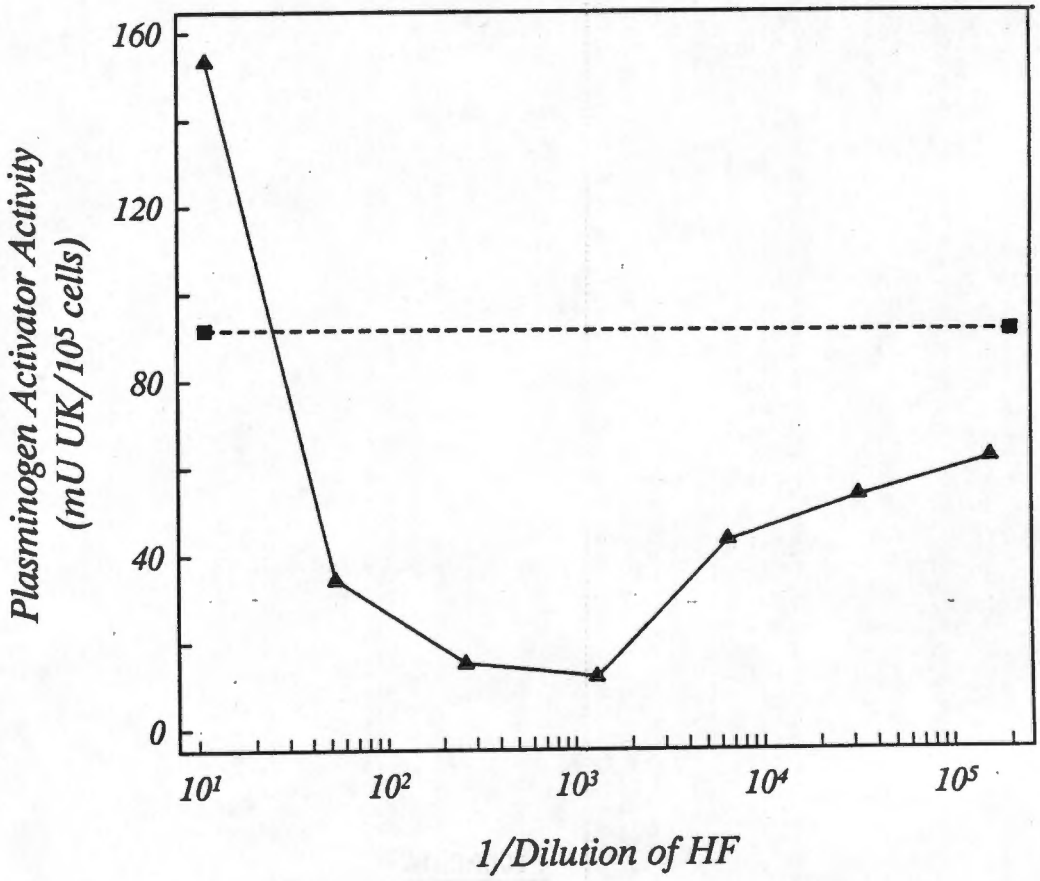

160
120
80
40
0
Plasminogen Activator Activity
(mU UK/10⁵ cells)
10¹
10²
10³
10⁴
10⁵
1/Dilution of HF

FIGURE 14

FIGURE 14

Effect of superinduced conditioned medium on plasminogen activator activity released by fibroblasts

Superinduced conditioned medium at the dilutions indicated was added to confluent fibroblast monolayers. After 48 hrs of incubation, the cultures were washed with DB and incubated for a further 24 hrs in serum-free DB. This conditioned medium was collected and assayed for plasminogen activator activity using a solid phase [^{125}I]-fibrin assay (Wilson and Dowdle, 1978).

Plasminogen activator activity in control cultures is indicated by the horizontal dashed line. Each point represents the average of the results obtained with 3 cultures.

Plasminogen activator (PA) activity released by fibroblasts treated with superinduced conditioned medium at a dilution of 1:10 was moderately elevated relative to that released by control cultures. A significant depression of PA activity in fibroblast harvest fluids at all higher superinduced conditioned medium dilutions was seen. No effect of superinduced conditioned medium on fibroblast cell number (approximately 2.5-3x10^5 per dish) was observed.

DISCUSSION

The results that I report in this chapter confirm other reports that have shown that collagen synthesis by confluent *in vitro* cultures of fibroblasts is a constitutive process that is, to a limited extent, inducible by the addition of resident murine peritoneal cells. Although induced rates of synthesis were no higher than 50% to 300% of unstimulated background levels, it should be noted that collagen turnover is a slow process *in vivo* (Woessner, 1968).

By making the simplifying assumptions that collagen breakdown is governed by a first-order rate constant K_d in the overall process that is described by the equation ($\delta C/\delta t = K_d C$) and that peritoneal cells induce an increase (ΔK_s) in a zero order synthetic rate constant K_s, it can readily be shown that the change (ΔC) in total collagen content at equilibrium is given by:

$$\Delta C = \Delta K_s / K_d$$

If, as is implied by a slow rate of turnover, the K_d is low, a small change (ΔK_s) in synthetic rate would result in a relatively large change (ΔC) in total collagen content at equilibrium. It is possible, therefore, that the magnitude of the stimulation in synthesis that is observed *in vitro* may have accounted for the extensive collagen deposition as seen *in vivo*.

I was consistently unable to confirm the reports of others (Hibbs et al., 1983; Jalkanen et al., 1979; Jalkanen and Penttinen, 1982; Matsushima et al., 1985) to indicate that mononuclear-cell conditioned medium contains soluble factors that induce collagen synthesis.

It occurred to me that the state of "activation" of the peritoneal cell population may have had something to do with my inability to demonstrate the release of fibroblast-stimulating factors, but experiments to examine this notion were unhelpful. Peritoneal exudate cells that had been activated in vivo by various inflammatory agents did not appear to be any more effective than resident cells as stimulators of collagen synthesis (Fig. 7). Medium conditioned by co-cultivated peritoneal cells and fibroblasts, in which in vitro activation may have taken place, was marginally and inconsistently stimulatory.

The effects of peritoneal cells on the kinetics of proline incorporation into collagen (Fig. 9) showed, consistently, that the addition of these cells increased the maximal rate of collagen synthesis but had no effect upon the apparent "Km" of the process. To the extent that one may draw detailed conclusions from overall results of this sort, I interpret these findings as indicating that co-cultivation with peritoneal exudate cells had no effect upon proline transport into the fibroblasts or upon the affinity constants involved in the enzymatic conversion of proline to collagen. The effect was rather to recruit a greater number of cells into collagen synthesis or to mobilise a greater fraction of the protein cellular

synthetic machinery for the manufacture of this particular protein. I hope to resolve this question by examining the cellular induction of collagen mRNA by in situ hybridization.

Murine macrophage responses to activating factors are known to be subject to genetic control: a fact that has been particularly well documented in studies on the responses of C3H/HeJ mice to lipopolysaccharide (Sultzer, 1968). Strain variation has also been reported for BCG-induced activation of tumoricidal cytotoxic macrophages (Boraschi and Meltzer, 1979) and in the development of non-specific resistance to pathogens (Civil and Mahmond, 1978; Medina et al., 1975). I accordingly felt that it would be of interest if I could show strain differences in the ability of murine peritoneal exudate cells to react to exposure to foreign fibroblasts by inducing collagen synthesis. Unfortunately (Fig. 8) the results of these experiments were uninteresting.

Attempts to obtain larger amounts of a putative "fibroblast-stimulating" factor by superinduction of P388D_1 cells led to the surprising discovery of a trypsin-sensitive, heat-stable, acid-labile substance that had a profound inhibitory effect upon collagen synthesis and upon the apparent release of plasminogen activator.

The inhibition of collagen synthesis was selective in that it did not depress overall radioactive leucine incorporation into other proteins. The reduction of plasminogen activator activity may have been due to

the induction of a plasminogen activator inhibitor such as PAI-1. This possibility has not been explored further.

The identity of the inhibitor(s) induced by superinduction of $P388D_1$ cells is unknown. Similar selective inhibition of collagen synthesis has been reported for TNF (Bertolini et al., 1986) and IFN (Rosenbloom et al., 1984; Stephenson et al., 1985). However IFN-γ, the most potent of the IFNs, is a T cell product. IFNα, although also effective but to a lesser degree (Jimenez et al., 1984), is usually acid stable. Therefore it is unlikely that the inhibitor is IFN. The possibility that the inhibitor is TNF needs still to be investigated.

$P388D_1$ cells were initially chosen in the hope that this would provide a constant expedient source of stimulatory activity for fibroblast collagen synthesis. The unexpected discovery of a potent inhibitor of collagen synthesis, although interesting, was not pertinent to the main theme of this thesis. I have thus left its further study to a later occasion.

CHAPTER 3

THE EFFECTS OF UCT-MEL 7 CELLS ON COLLAGEN SYNTHESIS BY FIBROBLASTS

In the previous chapter, I presented data to show that the induction of a desmoplastic response to melanoma cells could be mediated indirectly by macrophages. In this chapter, I address the question of whether or not tumour cells are able to interact directly with mesenchymal cells to elicit the deposition of extracellular matrix.

The idea that neoplastic cells may influence the quantity and composition of ECM in the environment is by no means new. Iozzo (1984) and Liotta (1982) have suggested three possible ways in which this may take place: by direct synthesis of matrix components; by degradation of the matrix; or by induction of synthesis of matrix components by host cells.

Generally speaking, malignant cells continue to produce matrix constituents characteristic of their normal untransformed state (Alitalo et al., 1981) but, in most cases, at a reduced rate (Hakomori et al., 1984; Sandmeyer and Bornstein, 1979). Qualitative and quantitative exceptions to these general rules have, however, been reported. For example, SV-40 transformed cells and clones selected for their high tumorigenicity produce an abnormal heparan sulphate with a reduced degree of sulphation (Winterbourne and Mora, 1981).

Furthermore, overproduction of hyaluronate (Biswas and Toole, 1987; Toole et al., 1979) or failure to produce or maintain a complete basal lamina (Ingber et al., 1981) are well documented quantitative abnormalities of ECM synthesis that have been correlated with tumour invasiveness.

There have also been reports of changes in the number of receptors for matrix components and in their cellular distribution. For example, neoplastic cells may show increased expression of laminin receptors which are no longer localized to the basal surface of the cell but are evenly distributed over the whole cell (Hand et al., 1985).

The ability of tumour cells to degrade the ECM may be regarded as a pre-requisite for invasion and metastasis if the solubilization of microbarriers to tissue penetration is necessary for these processes to occur. Some tumours have been shown to synthesize increased amounts of proteases - such as Type 1- and Type 4- collagenases (Liotta et al., 1980; Woolley et al., 1980), endoglycosidases (Fiszer-Szafarz and Gullino, 1970; Kramer et al., 1982) or cathepsin B (Poole et al., 1978; Sloane et al., 1981) - that degrade matrix components directly. Other neutral proteases, such as plasminogen activators, may increase degradation indirectly by activating latent collagenases (Werb et al., 1977).

Other tumours have the capacity to induce host mesenchymal cells to secrete matrix-degrading proteolytic enzymes. Basal cell carcinomas, for example, have been shown (Bauer et al., 1977; 1979) to contain

high levels of collagenase, which is usually localized to the stroma and is not found associated with the tumour cells themselves. An explanation for this phenomenon is provided by the finding that extracts of basal cell carcinomas contained a 19 kD cytokine that stimulated collagenase synthesis by fibroblasts (Goslen et al., 1985). Biswas et al. (1982; 1985; 1987) have identified a collagenase-stimulatory protein that is localized in the plasma membrane of B16 melanoma cells. This interacts with fibroblasts (possibly with heparan sulphate molecules in the fibroblast-associated matrix) and leads to the induction of collagenase synthesis. Similarly, rabbit V2 carcinoma cells induce the synthesis of cathepsin B (Graf et al., 1981).

Finally, tumours may modulate their environment by inducing the formation of excessive connective tissue around the neoplasm. The mechanisms involved are poorly understood but the evidence to date suggests that host stromal cells are induced by tumour cells to synthesize increased amounts of ECM components. Increased amounts of collagen have been documented in human breast carcinomas (Barsky et al., 1982) and gastric carcinomas (Minamoto et al., 1988), in rat mammary carcinomas (Bano et al., 1983), in V2 carcinoma in rabbits (Haemmerli et al., 1985) and in BL6 melanoma grown in 18 month old C57BL/6 mice (Barsky and Gopalakrishna, 1987). Other matrix components that show similar increases include proteoglycans (Haemmerli et al., 1985; Iozzo and Muller-Glauser, 1985; Iozzo et al., 1982) and elastin (Martinez-Hernandez and Catalano, 1980).

Increased levels of protease inhibitors may also contribute to the accumulation of matrix e.g. desmoplastic stroma surrounding human breast carcinomas contain high levels of inhibitors of both Type I and Type IV collagenases (Barsky and Gopalakrishna, 1986).

The idea that cells can influence adjacent cells is not limited to tumour:host interactions but occurs within normal situations as well. Cell-cell interactions are important for the development of hormonal responsiveness. This has been shown for the mammary gland (Heuberger et al., 1982; Haslam, 1986), retinal tissue (Saad et al., 1981) and embryonic urinary bladder (Cuhna et al., 1980). Co-operative cellular interactions are also involved in regulating the synthesis of compounds such as prostaglandins in the endometrium (Gal et al., 1982); collagenase in fibroblasts (Johnson-Wint and Gross, 1978; 1984) and proteoglycan synthesis and secretion by various cell types (Merrilees and Scott, 1980; 1981).

For the most part, the inductive effect of neoplastic cells on mesencymal macromolecular synthesis has been deduced from the results of <u>in vivo</u> studies: very few attempts have been made (Knudson et al., 1984; Merrilees and Findlay, 1985; Naito et al., 1984) to elucidate the mechanisms involved at a cellular or molecular level using an <u>in vitro</u> approach.

The experiments that I report in this chapter were designed to use this approach by co-cultivation of melanoma cells and fibroblasts and noting the effects of such co-culture on the rate of fibroblast

collagen synthesis. The results have shown that UCT-Mel 7 cells were consistent in their ability to stimulate fibroblast collagen synthesis by a mechanism that induced increased intracellular levels of collagen mRNA and that had an absolute requirement for intimate cell-cell contact. Elements of the ECM appeared to be important as mediators of this effect: soluble factors did not appear to be involved.

MATERIALS AND METHODS

Cells

Cell lines used in this chapter are:

Human cells:

-four melanoma cell lines: UCT-Mel 1, UCT-Mel 2, UCT-Mel 7 and RPMI 7272 (Bowes)

-four breast carcinoma cell lines: MCF 7, ZR-75-1, MDA-231 and T47-D

-foreskin fibroblasts

Animal cells:

-mouse fibroblast line, 3T3-J2

-rat embryo skin fibroblasts

-Chinese hamster fibroblast line, Don

Details of the cell lines are presented in the appendix.

Co-culture of UCT-Mel 7 cells and fibroblasts

Foreskin fibroblasts were plated at $1x10^5$ cells/35mm dish in DB-10 and grown to confluence (3 days). At confluence, UCT-Mel 7 cells

($3x10^5$/35mm dish) were plated on top of the fibroblasts in RPMI-10 and the cells were co-cultured for 24 hours. The next day, the medium was changed to serum-free RPMI containing 25μg/ml ascorbate and 220μg/ml proline (Merck 7434). The cultures were preincubated for 4 hours and then labelled for 16 hours with 5μCi [^{3}H]-Proline. The medium was collected and assayed for the incorporation of radioactivity into newly synthesized collagen as described in the methods section of Chapter 2. The results are expressed as μmoles proline/10^8 cells/16h.

Effects on fibroblast proliferation were determined by counting viable fibroblasts released from the dish by trypsinization. It was not possible to distinguish between fibroblasts and UCT-Mel 7 cells in the haemocytometer. All data presented for co-cultured fibroblasts were therefore calculated using the fibroblast cell number on the assumption that UCT-Mel 7 cells had no effect on fibroblast proliferation.

Where the effects of various compounds were investigated, the compounds were added together with the melanoma cells at the start of the 24 hour incubation and were presented throughout the duration of the experiment.

Monoclonal Antibody Production

Monoclonal antibodies were raised against UCT-Mel 7 cells and against foreskin fibroblasts. BALB/c mice were immunized with $4x10^6$ UCT-Mel 7 cells or $3x10^6$ fibroblasts. The immune spleens were removed and the splenocytes fused with SP2 myeloma cells using standard hybridoma

fusion techniques (Campbell, 1984). The final clone selected and used to make ascitic fluid was specific to the cell type against which it was raised. The monoclonal antibody, αFIB-D7, recognized fibroblasts and not UCT-Mel 7 cells. The monoclonal antibody, αNM-B12, was specific for UCT-Mel 7 cells and did not recognize fibroblasts. The characteristics of the 2 monoclonal antibodies are summarised in Table 1.

Conjugation of antibody to erythrocytes

The monoclonal antibodies were conjugated to red blood cells according to the method of Parish and McKenzie (1978). Human or sheep erythrocytes were washed 4 times in saline. To 4 ml saline, containing up to 1.6 mg of the appropriate antibody, was added 250μl of packed red cells. To this was added 0.4 ml of a 0.1% chromic chloride (BDH 10078) solution. The reaction was stopped after 5 minutes at room temperature by the addition of 7 ml of phosphate buffered saline (PBS). The conjugated erythrocytes were washed once in PBS and resuspended in 12.5 ml RPMI-10. They could be kept at 4°C for up to 24 hours before use.

Separation of a mixed cell population using conjugated erythrocytes.

The conjugated red blood cells were used to separate a mixture of UCT-Mel 7 cells and fibroblasts. After 24 hours co-culture, the cells were released with trypsin, resuspended in RPMI-10, mixed with an equal volume of conjugated red blood cells and incubated at room temperature for 15 minutes. The cell mixture was layered onto a Ficoll-Hypaque cushion and spun at 1000g for 15 minutes. The pellet

TABLE 1

Characteristics of the monoclonal antibodies

	αFIB-D7	αNM-B12
Isotype	IgG_{2a}	IgG_1
Isoelectric point	pH 7.3-7.8	pH 7.5
Dissociation constant	ND	$3x10^{-9}$ M
No. of sites/cell	$2.7x10^5$	$6.5x10^6$
Antigen recognized:		
Molecular weight	112kD	27kD
Sensitive to:		
SDS	Yes	No
Trypsin	No	No
Tunicamycin	No	No
Neuraminidase	No	No

contained the conjugated red blood cells and the cells recognized by the antibody conjugated to erythrocytes. The interface contained the cells not recognized by the antibody. The cells from the interface could be harvested and used in further experiments. A rapid and efficient separation of the 2 cell types was thus achieved.

Extraction of RNA and Northern blot analysis

Total RNA was extracted using guanidinium thiocyanate (Fluka 50990) as described by Chirgwin et al. (1979). The quality and quantity of the extracted RNA was checked by spectrophotometric analysis at 260nm and 280nm.

Twenty micrograms of RNA was heat denatured in formamide (Merck 9684) and electrophoresed in a 1.5% agarose gel containing 2.8M formaldehyde (BDH 10113) and transblotted electrophoretically onto nitrocellulose paper (Hybond N, Amersham RPN 303N) at 300mA for 16 hr in 25mM sodium phosphate buffer pH 6.5 (Amersham booklet: Membrane transfer and detection methods). The RNA was fixed to the Hybond by a 5 min exposure to UV light on a standard UV transilluminator.

The recombinant plasmids, Hf 677 (Chu et al.,1982) and Hf 32 (Myers et al.,1981), containing cDNA sequences specific for human α1(I) collagen gene and α2(I) collagen gene, respectively, were the gift of Dr. F. Ramirez, Rutgers Medical School, Piscataway, N.J. The recombinant plasmid, pHF βA-1 (Gunning et al.,1983), containing cDNA sequences specific for human cytoplasmic β-actin, was the gift of Dr. I. Parker, University of Cape Town Medical School. The cDNA plasmid probes were

nick-translated with [^{32}P]-dCTP (Amersham PB 10205) using the Amersham nick translation kit (Amersham N5000). The nitrocellulose filters were incubated in prehybridisation buffer for 6 hr at 55°C. The nick-translated probes were denatured at 100°C for 5 min, cooled, and added to the prehybridisation solution. Following hybridisation for 16 hr at 55°C, the RNA blots were washed extensively with washes of increasing stringency. The final wash was in 0.5X SSPE containing 0.1% SDS at 68°C for 15 min. The blots were then wrapped in Saran wrap and put up for autoradiography at -80°C using X-ray film.

Measurement of macromolecular synthesis

After a 5hr pulse with [^{3}H]-Leucine (5μCi/ml), [^{3}H]-Uridine (5μCi/ml;1μg/ml) or [^{3}H]-Thymidine (5μCi/ml;1μg/ml) the cultures were washed with PBS. The cells were released with trypsin and counted in a haemocytometer. Labelled macromolecules were then precipitated by the addition of ice-cold TCA (5% final conc). The TCA precipitate was collected on a filter and washed with ice-cold 5% TCA. The filter was dried overnight in a hot oven and the radioactivity measured in a liquid scintillation counter.

RESULTS

In the previous chapter, I reported experiments which showed that co-cultivation of fibroblasts and resident peritoneal exudate cells would induce fibroblast collagen synthesis *in vitro*. To look for a direct effect of melanoma cells, I co-cultivated melanoma cells and

human foreskin foreskin fibroblasts in the presence of [^{3}H]-Proline and obtained the results summarised in Fig. 1.

Melanoma cells alone synthesized negligible amounts of collagen (0.1 μmoles proline/10^8 cells/16 hr); fibroblasts alone incorporated 1.09 ± 0.1 μmoles proline/10^8 cells/16 hr. A mixture comprising of $3x10^5$ fibroblasts and $3x10^5$ melanoma cells incorporated 2.02 ± 0.2 μmoles proline/10^8 cells/16 hr; had there been no interactive stimulation this mixture should have incorporated 1.19 μmoles proline/10^8 cells/16 hr. This synergistic effect was consistently seen in a total of 46 experiments. The differences observed were highly significant.

Fibroblasts are known to produce both Type I and Type III collagen. Co-culture with UCT-Mel 7 cells induced increased synthesis of both Type I and Type III collagen (Fig. 2).

In view of the well known fact that cells may interact <u>in vivo</u> and <u>in vitro</u> by paracrine mechanisms, I examined melanoma cell conditioned medium for the presence of factors that might influence the fibroblast collagen synthesis. These experiments (Table 2) showed consistently that the medium alone was devoid of stimulatory activity. A number of experiments were performed to exclude trivial explanations for my inability to detect a stimulatory factor:

(i) The possibility that the released factors were labile was rendered unlikely by taking precautions to preserve the conditioned medium

FIGURE 1

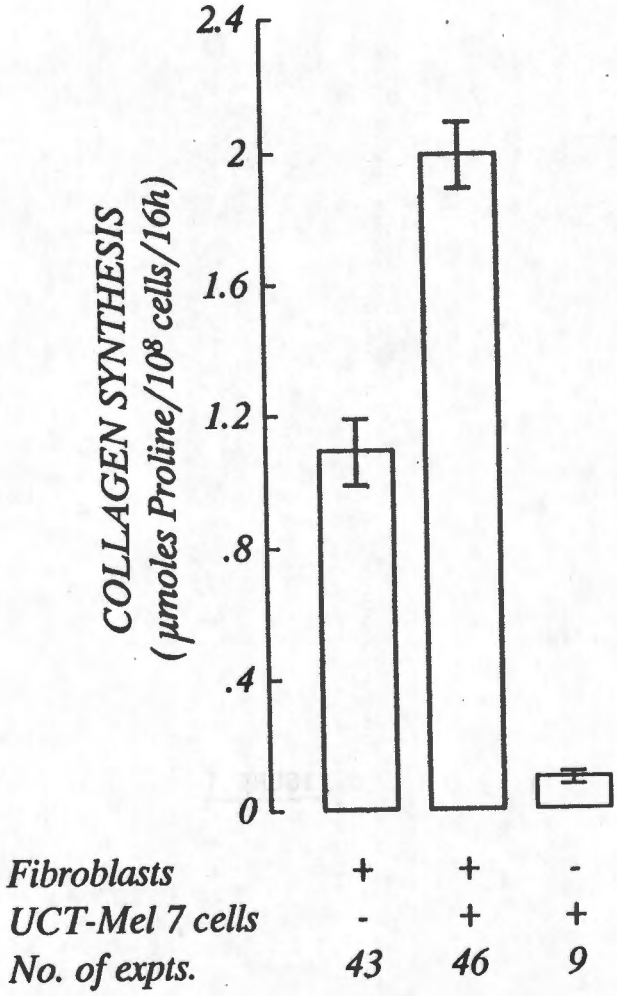

COLLAGEN SYNTHESIS
(μmoles Proline/10⁸ cells/16h)
2.4
2
1.6
1.2
.8
.4
0
Fibroblasts + + -
UCT-Mel 7 cells - + +
No. of expts. 43 46 9

FIGURE 1

FIGURE 1

Effect of UCT-Mel 7 cells on fibroblast collagen synthesis

This figure depicts the results obtained in replicate experiments in which fibroblasts and melanoma cells were cultured alone or together and the rate of collagen synthesis was measured. In all cases the cells were incubated in 35mm dishes under 1 ml of RP-10 for a preliminary period of 24 hrs after which medium was removed and replaced with 1 ml of serum-free RPMI containing 5 μCi [^{3}H]-Proline (2.087 mM). Radioactive collagen synthesized during this period and released into the medium was measured as described in the text.

Fibroblasts cultured alone (43 experiments; triplicate dishes) were studied when they had reached confluence. Melanoma cells (9 experiments; triplicate dishes) were plated at $3x10^5$ per dish and showed little increase in cell number over the ensuing 40 hrs. In the co-cultures (46 experiments; triplicate dishes) UCT-Mel 7 cells ($3x10^5$ per dish) were plated over confluent fibroblasts at the start of the preliminary 24 hr incubation period.

The height of each bar represents average values for the collagen synthesis measured in terms of μmoles proline incorporated/10^8 cells/16 hr. Error bars represent 1 s.e.m.

The approximately 2-fold induction of collagen synthesis by the melanoma cells was highly significant $p < 0.001$.

FIGURE 2

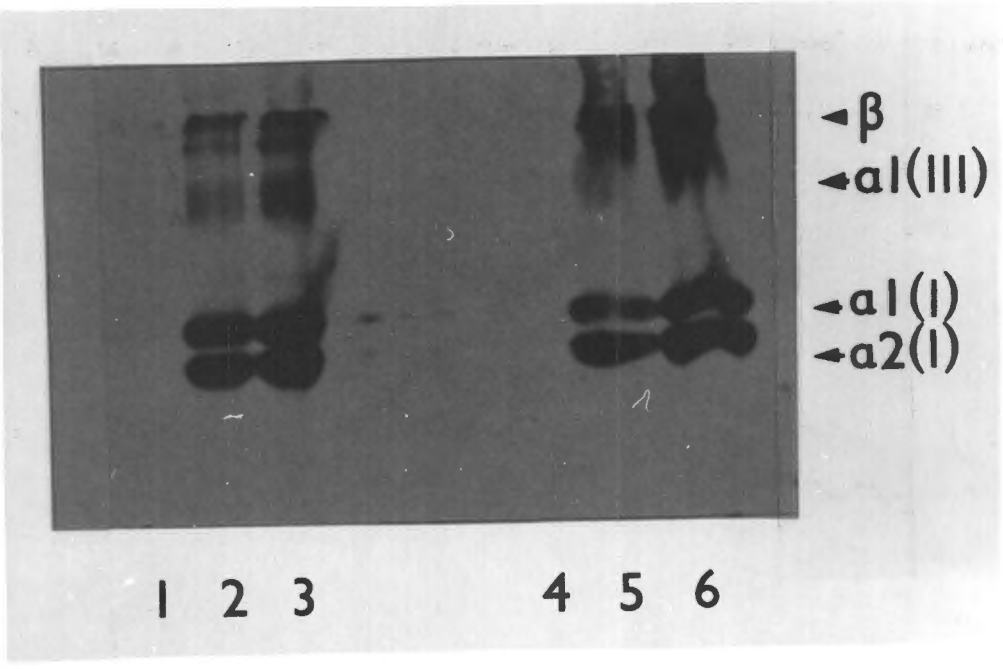


FIGURE 2

FIGURE 2

Type of collagen produced by fibroblasts cultured alone or with melanoma cells

Fibroblasts and melanoma cells were cultured alone or together and labelled with [^{3}H]-Proline as described in the legend to Fig. 1 save for the addition of 50 μCi of radioactive amino acid instead of 5μCi. At the end of incubation the radioactive collagen was pepsin treated and purified by two successive salt precipitations as described in the Methods section. The final precipitate was dissolved in 0.2M acetic acid and electrophoresed in a 7% polyacrylamide gel containing 0.1% SDS with (lane 1-3) or without (lanes 4-6) reduction. After electrophoresis the gel was dried and exposed to autoradiographic film for 16 hrs.

Labelled arrow heads indicate the typical mobilities of the α chains of Type I and Type III collagen (the latter only seen in reducing gels) and the so-called β chain that represents a multimeric form of the α chains (Miller and Rhodes, 1982).The patterns obtained with melanoma cells alone (lanes 1 and 4; no visible collagen synthesis), fibroblasts alone (lanes 2 and 5) and fibroblasts and melanoma cells (lanes 3 and 6) are shown.

Note that the fibroblasts synthesized mainly Type I collagen with small amounts of Type III. The apparent induction of collagen synthesis by the melanoma cells, as indicated by the more intense radioactive bands, although probably real is not quantitatively reliable since internal standards were not included in this qualitative experiment that was designed to identify the type of collagen synthesized.

TABLE 2

TABLE 2

Lack of effect of UCT-Mel 7 cell-conditioned medium (CM)[1] on fibroblast collagen synthesis[2]

Expt No.	Cell No. ($x10^{-5}$)	Addition	[^{3}H]-Proline incorporation into collagen (dpm or μmoles/10^8 cells/16hr)
1[3]	5.55	RPMI	0.981μmoles
	5.65	7xconc CM	0.866 "
	5.55	$3x10^5$ UCT-Mel 7 cells	1.673 "
2	3.34	RPMI	31 729 dpm
	3.21	CM	37 682 "
3	5.27	RPMI	88 571 dpm
	5.32	CM	92 416 dpm

Footnotes to Table 2

1. Conditioned medium was prepared by plating UCT-Mel 7 cells at $3x10^5$/35mm dish in RP-10 and allowing them to reach semi-confluence. The cells were then washed with serum-free RPMI, covered with 1 ml of RPMI and incubated for 24 hr. At the end of this time the medium was collected, centrifuged to remove debris and particulate material and used immediately in experiments 2 and 3. For experiment 1, the medium was concentrated 7-fold with an Amicon stirred pressure cell using an ultrafiltration membrane with nominal molecular weight cut-off of 10 kD. The retentate was then used in the experiment.

2. The effects of media on fibroblast collagen synthesis were measured by adding, in each case, medium without added serum to 35mm dishes containing confluent fibroblast monolayers. After 24 hr [^{3}H]-Proline (10μCi/dish) was added and incubation continued for a further 16 hr. The radiolabelled collagen was then isolated and counted as described in the Methods section.

3. In this experiment, non-radioactive proline was added to the medium to a final concentration of 2.087mM to ensure that the concentration of the precursor amino acid would not be rate-limiting (cf. Fig. 6). In the other two experiments the proline concentration was that of the RPMI serum-free medium (0.174mM).

under conditions of temperature and sterility that would favour preservation and by experiments in which fibroblasts and melanoma cells were co-cultivated in Cooper dishes in which the cells shared the same medium without being contiguous. In these experiments fibroblasts were plated on the bottom of the dish and the melanoma cells were plated on the under surface of the depressed lid (Fig. 3) in such a way that the two monolayers were separated by 4 mm. The results (Table 3) showed no effect of the melanoma cells on the fibroblasts. When the cells were co-cultivated under the same conditions but intimately mixed stimulation of collagen synthesis was seen.

(ii) The medium was examined for the presence of collagenase that might have degraded released collagen. None was found.

(iii) The possibility that stimulatory factors were released at too low a concentration to stimulate without the cumulative effect of prolonged exposure of the target cell to the factors was explored by testing a seven times concentrate of the melanoma cell conditioned medium. The concentrate was prepared in an Amicon stirred cell using a YM10 membrane with a nominal molecular weight cut-off of 10 kD (Amicon Corporation). A concentrate prepared in this manner had no effect (Table 2).

(iv) The possibility that the release of fibroblast stimulatory factors by melanoma cells was not constitutive but only

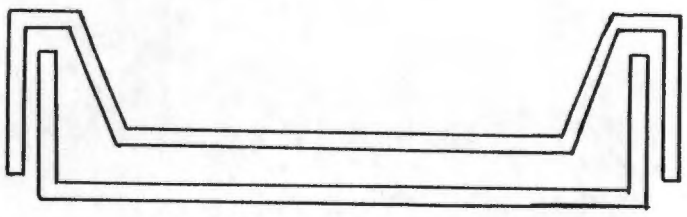


FIGURE 3

Diagrammatic representation of a Cooper dish

The Cooper dish (Falcon Cat. No. 3009) is designed for the co-cultivation of cells such that they share the same medium but are not contiguous. Cells may be plated in the conventional manner on the bottom of the petri dish and also on the undersurface of the indented lid. Co-culture is then established by the simple expedient of transferring a lid containing one cell type to a bottom with the other cell type as an adherent monolayer. Sufficient medium is then added to ensure that both monolayers are adequately covered.

TABLE 3

TABLE 3

Cooper dish experiments[1]

Expt No.		Experimental Conditions[2] (Cell No. x 10^{-5})		[^{3}H]-Proline incorporation into collagen[3] (dpm/10^5 cells/24hr)
		Bottom	Lid	
1	a)	UCT-Mel 7(10)	Fib (3)	146 500
	b)	-	Fib (3)	170 200
	c)	UCT-Mel 7(10)	-	2 400
2	a)	UCT-Mel 7(10)	Fib(2.6)	51 073
	b)	-	Fib(2.6)	70 859
	c)	UCT-Mel 7(10)	-	6 800
3	a)	UCT-Mel 7(10)	Fib (2)	27 327
	b)	-	Fib (2)	21 043
4	a)	Fibs (2)	UCT-Mel 7(2.5)	53 716
	b)	Fibs (2)	-	51 524
5	a)	Fibs (1)		47 855
	b)	Fibs (1)+ UCT-Mel 7(1)		110 540
6	a)	Fibs (1)		14 213
	b)	Fibs (1) + UCT-Mel 7 (1)		45 580
7	a)	Fibs(1)		26 291
	b)	Fibs(1) + UCT-Mel 7(1)		61 173

Footnotes to Table 3

1. In these experiments co-cultivation of fibroblasts and UCT-Mel 7 cells was achieved by plating one cell type on the bottoms and the other on the under-surface of the indented lids of Cooper dishes (Falcon Cat. No. 3009; see Fig. 3). Under these experimental conditions the two cell types shared the same medium separated by approximately 4mm without cell:cell contact.

2. In experiments 1-4, cultures were established by plating the requisite number of cells of each type on separate dishes, on bottoms or lids as indicated. After the cells had adhered, co-culture was established by the simple expedient of transferring a lid containing one cell type to a bottom with the other cell type as an adherent monolayer and sufficient medium to ensure that both monolayers were adequately bathed. After 24 hrs of co-culture the medium was changed and the cells were co-cultivated in the presence of medium containing [^{3}H]-Proline for a further 24 hrs. Radioactive collagen released into the medium was then measured as described in the Methods section.

   In experiments 5-7, fibroblasts were plated alone or mixed with UCT-Mel 7 cells. The number of each cell type plated is given in parentheses.

3. The results in this table are given as radioactive proline incorporated into collagen rather than μmoles incorporated since the kinetic experiments (Fig. 6) had not yet been performed; no unlabelled proline was added to the medium and the specific activity of the precursor proline was uncertain.

induced by contact with fibroblasts was investigated by testing conditioned medium taken from co-cultures of melanoma cells and fibroblasts plated at ratios known to cause stimulation. Such conditioned medium had no effect.

The kinetics of induction of collagen synthesis by co-cultivation with melanoma cells were studied in the experiments summarized in Fig. 4. The increase in the rate of collagen synthesis that was measured as a function of melanoma:fibroblast ratio showed a progressive increase up to a maximum of a two-fold stimulation observed at a ratio of melanoma cells:fibroblasts of 1:1. Higher ratios than this were less stimulatory.

The addition of melanoma cells to confluent fibroblast monolayers caused an increase in the rate of collagen synthesis that was evident by the time the first time point was taken four hours after initiation of co-culture. Incorporation of [^{3}H]-Proline into collagen continued at the same increased rate for the remainder of the duration of the experiment (Fig. 5).

Induction of collagen synthesis by melanoma cells implied the existence of rate-limiting steps for the incorporation of proline into collagen in unstimulated fibroblasts. To obtain some idea of the nature of the kinetic constraints that were overcome by contact with melanoma cells, I performed the experiment in which I measured fibroblast collagen synthesis as a function of proline concentration in the presence and absence of melanoma cells. The

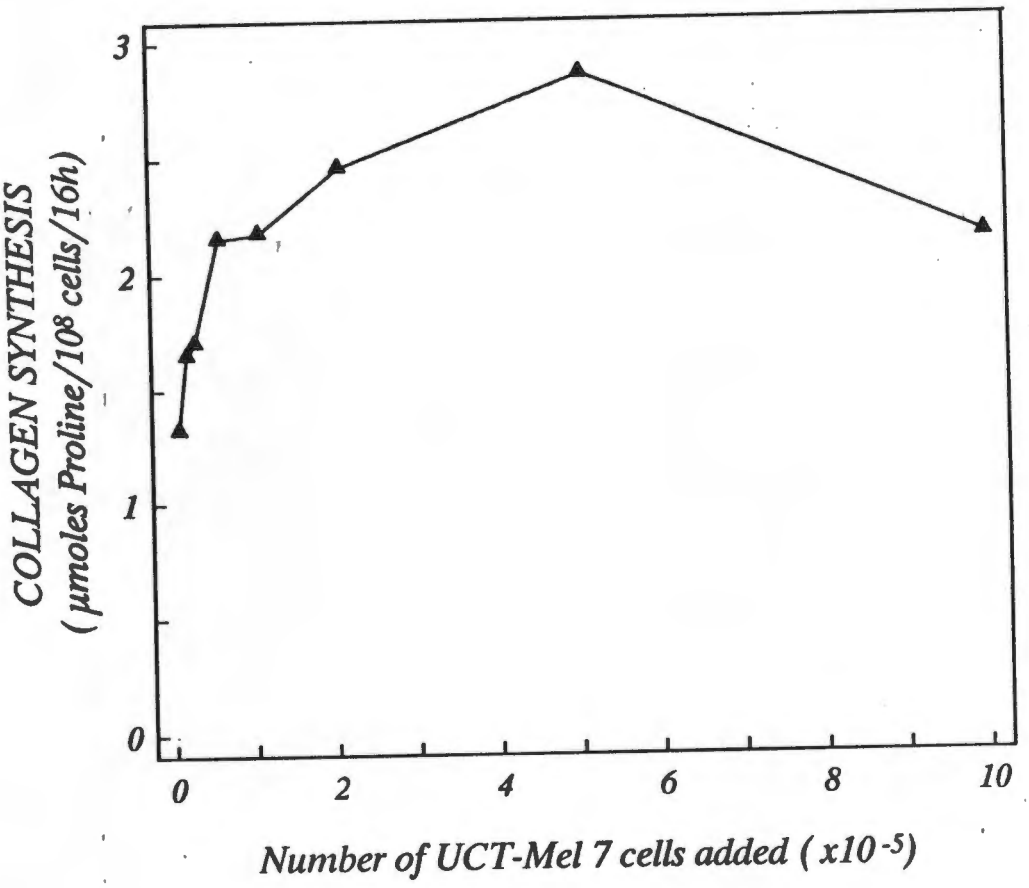


FIGURE 4

Dose-dependent stimulation of collagen synthesis by melanoma cells

Increasing numbers of UCT-Mel 7 cells were plated on top of confluent fibroblast monolayers (approximately $5x10^5$/35mm dish) and co-cultured for 24 hrs. The cultures were then labelled for 16 hr with 5μCi [^{3}H]-Proline and the supernatant processed for the measurement of radiolabelled collagen. Each point represents the average of the results of 3 dishes.

Increasing numbers of UCT-Mel 7 cells resulted in an increase in the stimulation of collagen synthesis by the fibroblasts. Maximal stimulation occurred at a melanoma:fibroblast ratio of 1:1.

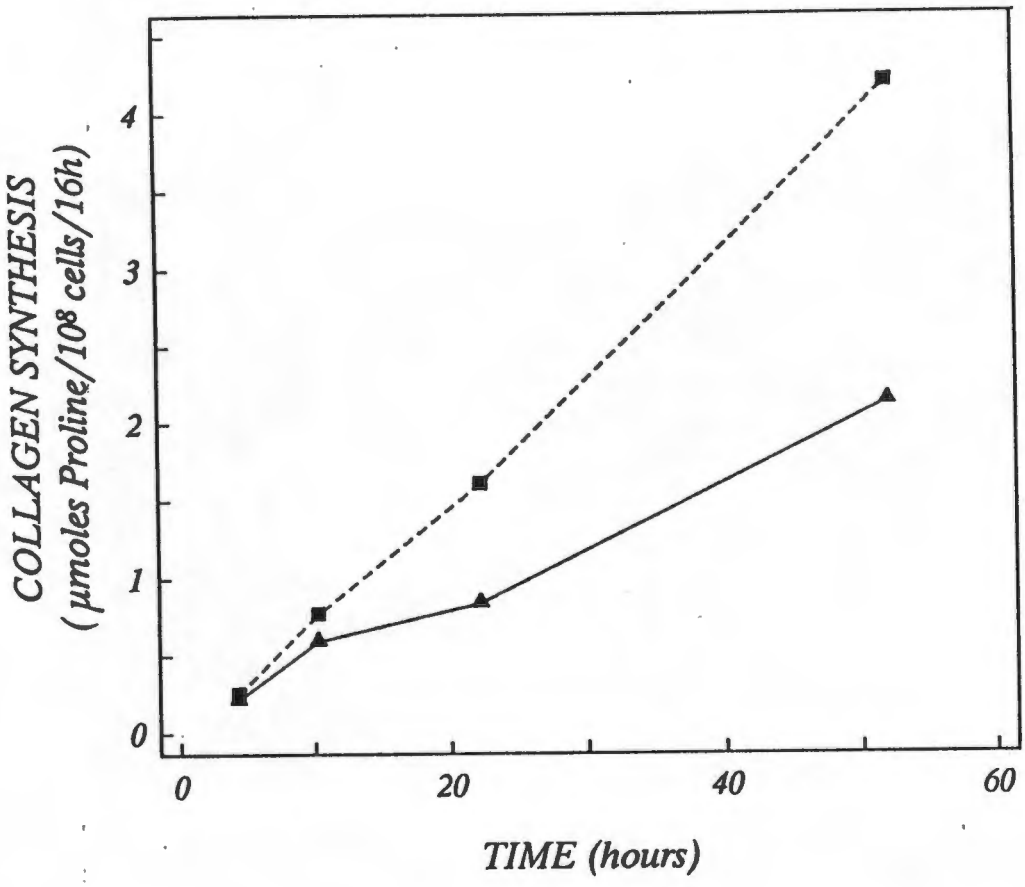


FIGURE 5

Co-culture of fibroblasts and UCT-Mel 7 cells: collagen synthesis as a function of time

Confluent fibroblast monolayers, either alone (▲——▲) or as co-cultures with melanoma cells (■----■), were labelled with 10μCi [^{3}H]-Proline and the culture supernatant harvested at the indicated time points for the measurement of radioactiave collagen. Co-cultures were established by plating UCT-Mel 7 cells (3x10^5 cells/35mm dish) on the confluent fibroblast monolayers at time 0.

Each point represents the average of the results of 3 dishes.

Incorporation of proline into collagen was linear over 52 hours. The rate of incorporation was greater in the co-cultured fibroblasts than in the fibroblasts alone.

results, analysed graphically by the method of Lineweaver and Burke, are presented in Fig. 6. These show quite clearly that the effect of melanoma cells was to increase the apparent maximum rate of proline incorporation into collagen from 1.99 μmoles/10^8 cells/16 hr to 4.89 μmoles/10^8 cells/16 hr. The apparent "Michaelis constant (Km)" for the reaction remained unchanged (1.08 mM in the absence and 1.01 mM in the presence of melanoma cells).

The effect of melanoma cells on fibroblast collagen synthesis was specific for collagen and not due to an increase in overall protein synthesis. Protein synthesis, as measured by [^{3}H]-Leucine incorporation, was studied in cells cultured alone or as co-cultures with melanoma cells. There was no stimulation of general protein synthesis in the fibroblasts that had been co-cultured with UCT-Mel 7 cells (Fig. 7).

<u>The effect of the melanoma cells on fibroblast collagen synthesis was not species specific.</u>

It is well known that the effects of certain growth factors, such as the interferons, are highly species specific. To test for similar specificity in my system, I examined the effect of the human melanoma cell line on collagen synthesis by fibroblasts derived from a number of other species. The results showed that the effect on human, mouse, rat and hamster cells were comparable (Table 4).

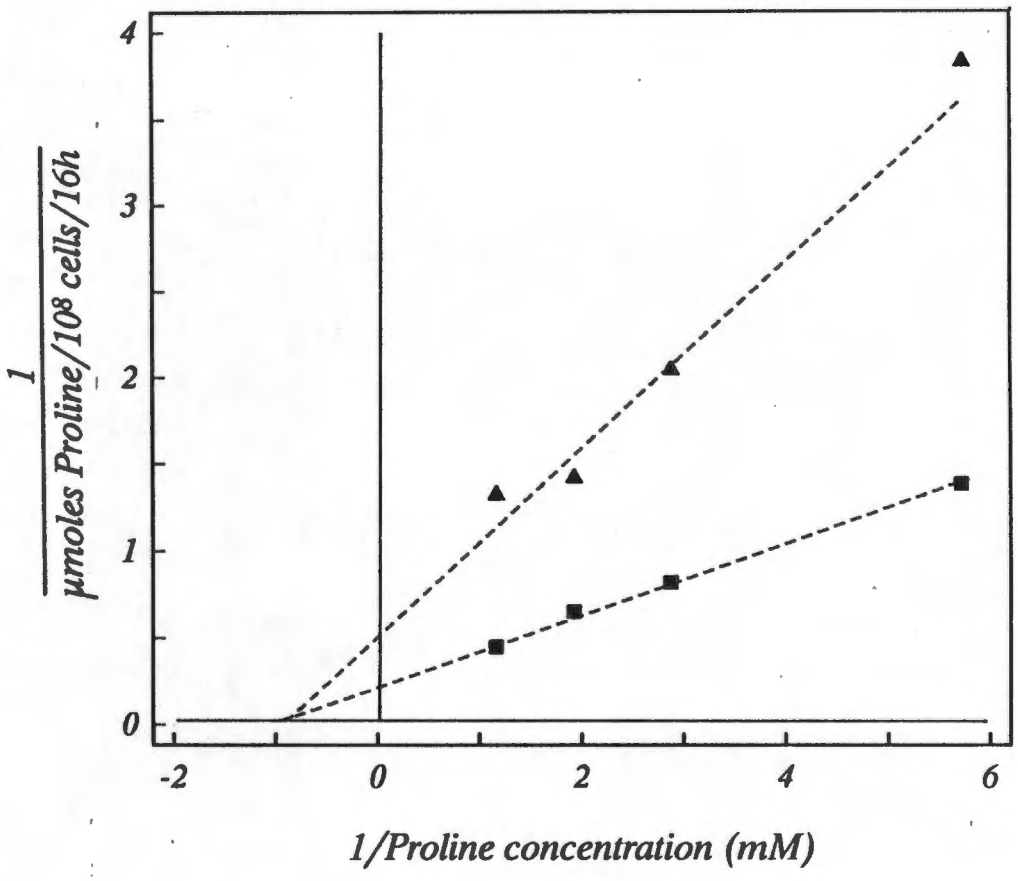


FIGURE 6

Lineweaver-Burk analysis of Proline incorporation

In this figure the reciprocal of the rate of proline incorporation into collagen (μmoles/10^8 cells/16 hrs) has been plotted as a function of the reciprocal of the proline concentration (mM) for graphic analysis according to the method of Lineweaver and Burk.

Fibroblasts cultured in the presence of UCT-Mel 7 cells ($3x10^5$/dish; ■----■) and those cultured alone (▲-----▲) showed similar apparent "Km" values (1.01 and 1.08 mM, respectively) but different extrapolated maximal rates of incorporation (4.89 and 1.99 μmoles/10^8 cells/16 hr, respectively).

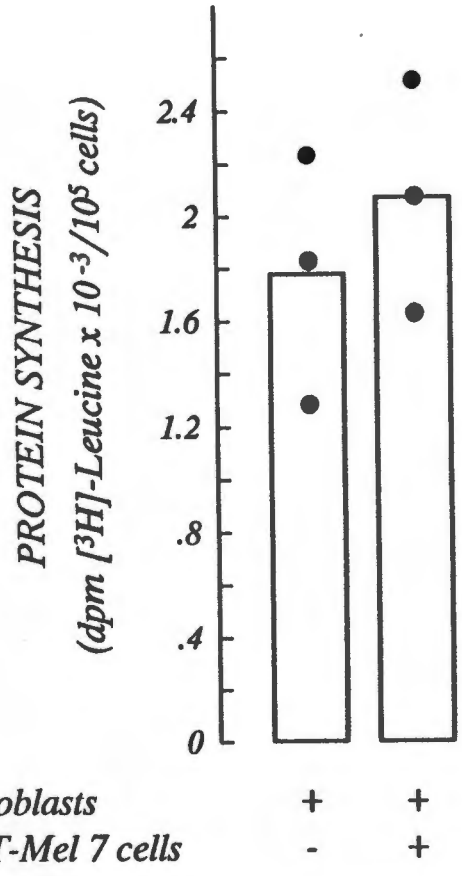


FIGURE 7

Effect of melanoma cells on protein synthesis

Confluent fibroblast monolayers either alone or as co-cultures with melanoma cells were incubated in RP-10 for 24 hrs after which they were labelled for 4 hrs with [^{3}H]-Leucine (5μCi/dish). The co-cultures were established by plating UCT-Mel 7 cells ($3x10^5$/35mm dish) on confluent fibroblast monolayers at the start of the 24 hr incubation. The cells were released with trypsin and the co-cultured cells separated using antibody-conjugated erythrocytes as described in the Methods section. Radiolabelled protein was precipitated with TCA (final concentration 5%), collected and counted as described in the Methods section.

Each point represents values for individual dishes; bar heights represent the average.

There was no stimulation of overall protein synthesis in fibroblasts that had been co-cultured with UCT-Mel 7 cells.

TABLE 4

Effect of UCT-Mel 7 cells on collagen synthesis by fibroblasts of different species

	Collagen Synthesis	
Source of fibs	Fibs alone	Fibs co-cultured
Human foreskin	1.09(±0.1)	2.02(±0.1)
Rat embryo skin	0.929(±0.21)	2.633(±0.37)
Chinese hamster (Don)	0.108(±0.01)	0.247(±0.03)
Mouse (3T3)	0.549(±0.11)	1.085(±0.31)

Confluent fibroblast monolayers were cultured either alone or as co-cultures with melanoma cells for 40 hr. For the last 16 hr of culture, 5μCi [^{3}H]-Proline was added. The supernatants were then processed for the measurement of collagen synthesis. The co-cultures were established by plating UCT-Mel 7 cells ($3x10^5$/35mm dish) on confluent fibroblast monolayers. The results are expressed as μmoles proline/10^8 cells/16 hrs. Values represent the average of the results of triplicate dishes ± 1 s.e.m.

The in vitro effect of melanoma cells on collagen synthesis was independent of passage number.

The desmoplastic responses of nude mice to inocula of UCT-Mel 7 cells in vivo showed a striking relationship to the melanoma cell passage number from which the inocula were prepared (Chapter 1). Cells from early passage numbers were not fibrogenic in vivo; desmoplasia was seen maximally with inocula of cells from 20 - 30th passage; cells taken from in vitro passage number >50 produced tumours that were too small during the dormant phase to evaluate desmoplasia. A similar correlation between passage number and induction of collagen synthesis in vitro was not observed. Melanoma cells from early, middle and late passages were equally effective in this regard (Fig. 8).

Other tumour cell lines also stimulated collagen synthesis but none as effectively as UCT-Mel 7.

The results of in vivo studies with other melanoma cell lines showed that UCT-Mel 7 was particularly effective as an inducer of a desmoplastic response (Chapter 1, Figure 9).

In view of this observation, I tested a number of other melanoma cell lines and cell lines derived from tumours that are known to elicit a desmoplastic response, for their ability to stimulate collagen synthesis in vitro. The results of these experiments (Fig. 9) showed that all of the cell lines tested increased collagen synthesis to a modest extent (30% - 130%). None, however,

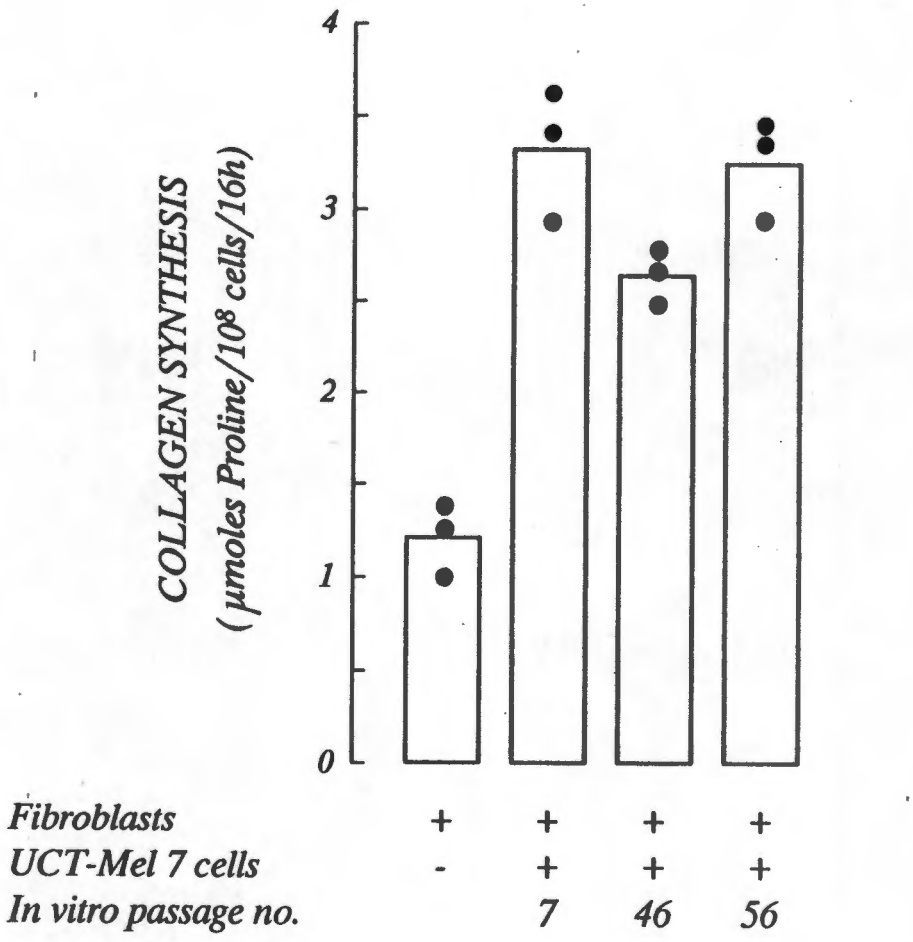


FIGURE 8

Stimulation of fibroblast collagen synthesis by UCT-Mel 7 cells: effect of in vitro passage number

UCT-Mel 7 cells ($3x10^5$/35mm dish) at in vitro passage numbers 7, 46 or 56 were added to confluent fibroblast monolayers. After 24 hours of co-culture the cells were labelled for 16 hrs with 5μCi [^{3}H]-Proline for the measurement of radiolabelled collagen. Average values (bar heights) and results for individual dishes (circles) are given.

No significant effect of the passage number was observed.

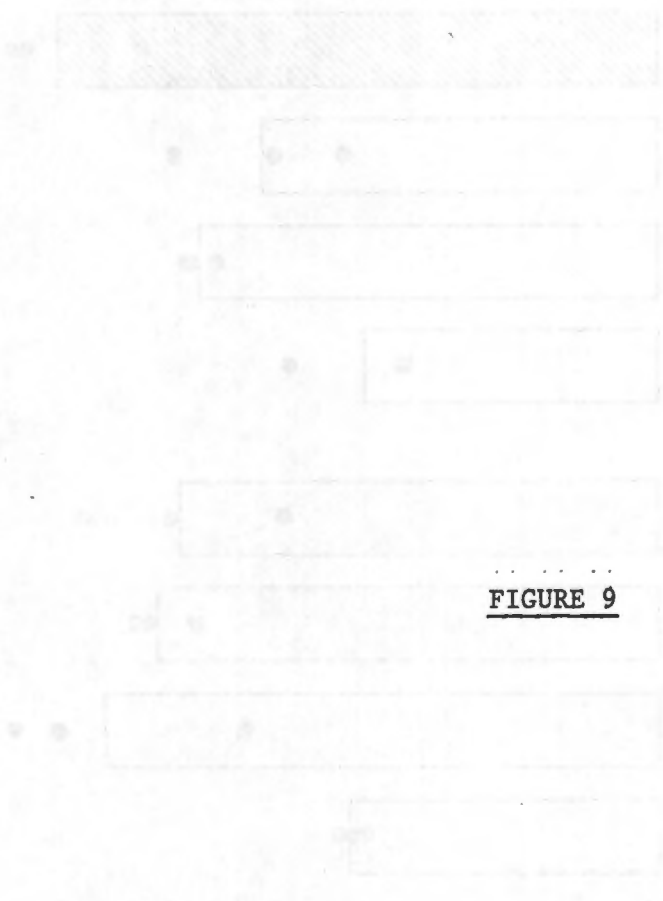


FIGURE 9

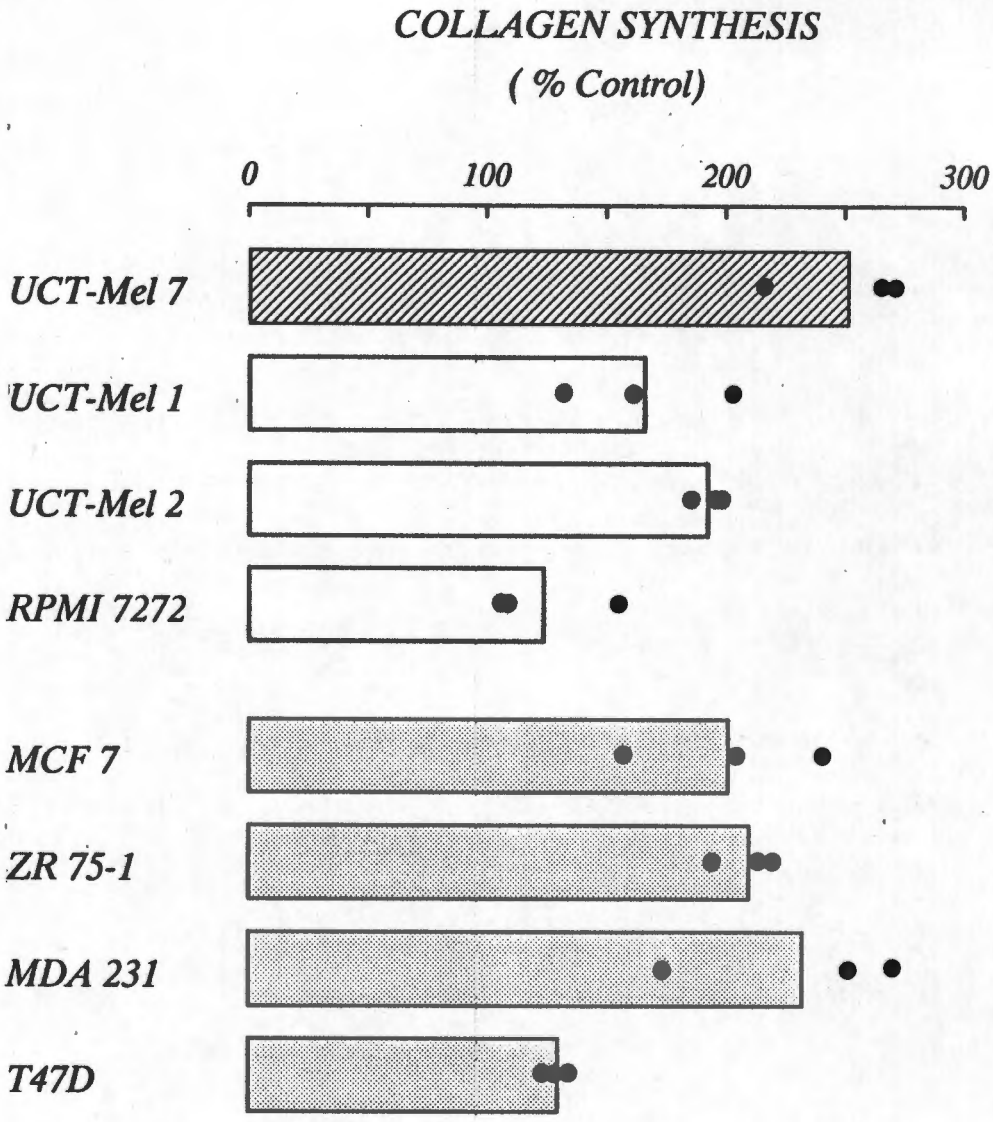

COLLAGEN SYNTHESIS
(% Control)
0
100
200
300
UCT-Mel 7
UCT-Mel 1
UCT-Mel 2
RPMI 7272
MCF 7
ZR 75-1
MDA 231
T47D

FIGURE 9

FIGURE 9

Effect of other cell lines on fibroblast collagen synthesis

Confluent fibroblast monolayers were cultured either alone or as co-cultures with cell lines to be tested. After 24 hr of co-culture, the cells were labelled for 16 hrs with 5μCi [^{3}H]-Proline for the measurement of collagen synthesis. Co-cultures were established by plating the tumour cells ($3x10^5$/35mm dish) on confluent fibroblast monolayers.

Each point represents the value for an individual dish of co-cultured cells expressed as a percentage of the average value for triplicate dishes, in the same experiment, in which fibroblasts were cultured alone. UCT-Mel 7, UCT-Mel 1, UCT-Mel 2 and RPMI 7272 are melanoma cell lines. MCF-7, ZR-75-1, MDA 231 and T47D are breast carcinoma cell lines.

Co-culture with other tumour cells induced variable stimulation of collagen synthesis. With the exception of MDA 231, none was as effective as UCT-Mel 7.

was as effective as UCT-Mel 7 which consistently stimulated to an extent >two-fold (Fig. 1).

Inhibition of protein synthesis but not of DNA synthesis interfered with melanoma cell stimulatory activity.

To investigate the extent to which the induction of collagen synthesis in fibroblasts required intact melanoma cell function, I pre-treated the melanoma cells with different inhibitors before adding the fibroblasts and noted the effects of such pre-treatment. In two experiments, UCT-Mel 7 cells that had been pre-treated with concentrations of mitomycin C that were sufficient to inhibit [^{3}H]-Thymidine incorporation completely, were nevertheless capable of inducing an increase in fibroblast collagen synthesis that was equal to or, slightly greater than, the stimulatory effect seen with untreated melanoma cells (Table 5). To investigate the need for melanoma cell mRNA synthesis, I pre-incubated cultures of UCT-Mel 7 cells with actinomycin D for one hour at concentrations ranging from 10μg - 2.5μg/ml. The cells were washed 3 times and fibroblasts added to measure the effect on collagen synthesis using the usual protocol. The concentration of actinomycin D required to inhibit the melanoma cell RNA synthesis were previously determined by titration (Fig. 10). In two separate experiments, I found that, despite extensive washing of the melanoma cell cultures, the actinomycin D treatment carried over a profound inhibitory effect on collagen synthesis to co-cultivated fibroblasts so that rates of incorporation of [^{3}H]-Proline into collagen were reduced to levels approximately 1/10 of the level seen with unstimulated fibroblasts.

TABLE 5

Effect of mitomycin C-treated UCT-Mel 7 cells on fibroblast collagen synthesis

		Collagen Synthesis (μmoles Proline/10^8 cells/16 hr)	
	Mit.C Conc (μg/ml)	Fibs alone	Fibs co-cult
Expt 1	None	1.423	1.879
	0.25		2.007
Expt 2	None	1.199	2.511
	0.5		3.022
	1.0		3.067

UCT-Mel 7 cells ($3x10^5$/35mm dish) were treated with mitomycin C for 18 hrs. The cultures were then washed with warmed RPMI (3x10 mins) and fibroblasts ($3x10^5$/35mm dish) were added. After 24 hr of culture the cells were labelled for 16 hr with 5μCi [^{3}H]-Proline and the supernatant processed for the measurement of radioactive collagen. Values represent the average of the results of triplicate cultures.

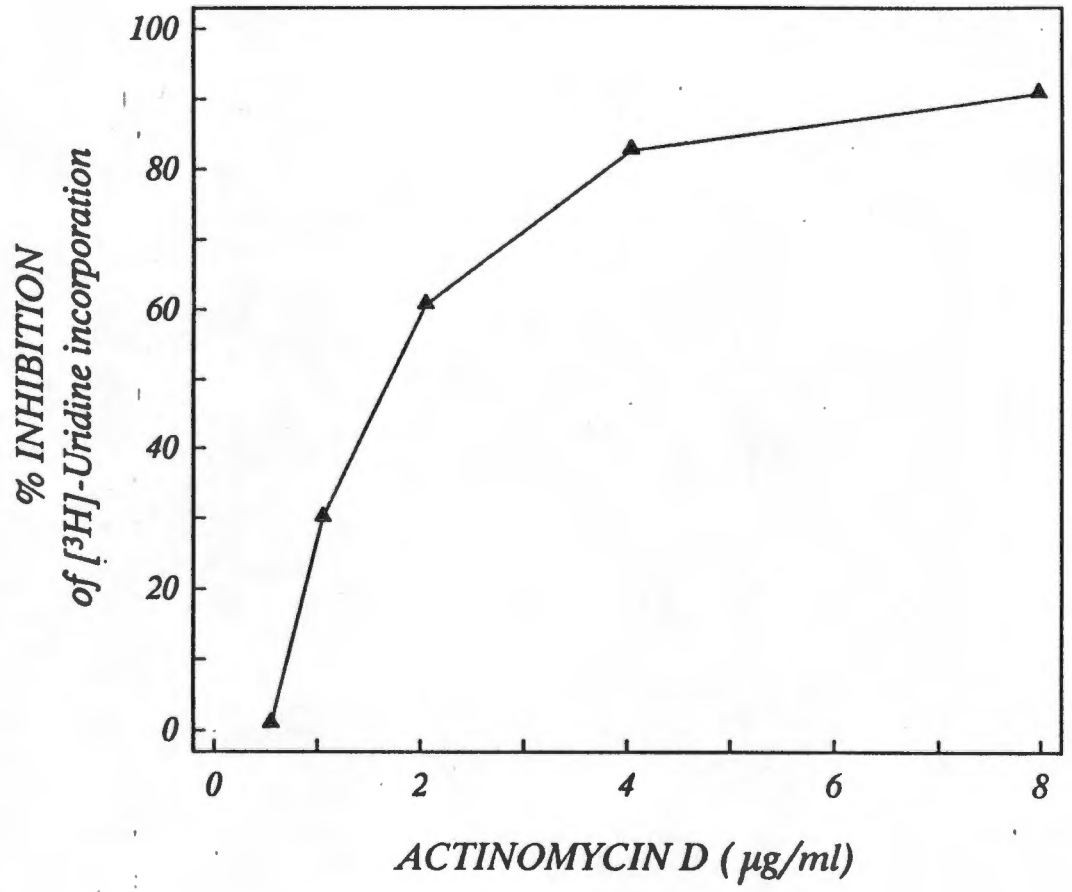


FIGURE 10

Inhibition of RNA synthesis: titration of actinomycin D

Semi-confluent UCT-Mel 7 cultures (± $3x10^5$ cells/35mm dish) were treated with actinomycin D for 1 hr at 37°C at the concentrations shown. The cultures were then washed 3 times with RPMI, incubated for 24hr in 1 ml RP-10, and for a further 4 hrs in the presence of 2μCi [^{3}H]-Uridine. The cells were released with trypsin and the TCA precipitable material determined as described in the Methods section.

Each point represents the average of the results of triplicate cultures expressed as percentage inhibition relative to untreated cultures.

It was not possible, therefore, with this inhibitor to obtain useful data in my co-cultivation system.

Cycloheximide is commonly used to evaluate the need for protein synthesis since this compound is a fairly specific inhibitor of peptidyl transferase, the ribosomal enzyme that forms peptide bonds (Alberts et al., 1983). Under other circumstances it would thus have been a useful reagent to establish the need for melanoma cell protein synthesis but, unfortunately, cycloheximide is a reversible inhibitor that would have been removed during the obligatory wash before the fibroblasts were added and thus have yielded equivocal results. Although it is commonly stated that puromycin is an irreversible inhibitor of protein synthesis, I found that puromycin at high concentrations ($>10^{-6}$M) was toxic to the cells. Exposure to concentrations of 10^{-6}M or less for 18hrs had no effect upon protein synthesis (Table 6). I, therefore, discarded puromycin as a potentially useful reagent.

In my quest for a more suitable inhibitor, it occurred to me that glutaraldehyde may prove valuable. This compound, known for its efficacy as a fixative, is also known to preserve cell surface enzymatic function (Poznansky, 1988) and, in many instances, to preserve antigenicity of labile cell surface epitopes (Adler et al., 1985). Since the requirement for melanoma cell:fibroblast contact implied the possible interaction of viable fibroblasts with melanoma cell surface molecular structures, I felt that I might be able to determine a glutaraldehyde concentration that would

TABLE 6

Effect of puromycin on UCT-Mel 7 protein synthesis

Puromycin conc.	[^{3}H]-Leucine incorporation (dpm/10^5 cells/5 hr)
None	7 502
$5x10^{-5}$M	4 097
10^{-6}M	8 719
10^{-7}M	7 490

UCT-Mel 7 cultures ($5x10^5$ cells/35mm dish) were treated with puromycin for 18 hr at the concentrations shown. The cultures were then washed, 3 times with RPMI, incubated for 24 hr in 1 ml RP-10 and a further 5 hrs in the presence of [^{3}H]-Leucine (2μCi/dish). The cells were then released with trypsin and the radioactive protein precipitated with TCA (5% final concentration), collected and counted.

Puromycin at concentrations > 10^{-6} M were toxic for the cells. At concentrations $\leq 10^{-6}$ M no effects on protein synthesis were seen.

preserve stimulatory capacity in melanoma cells that were no longer viable. I therefore studied the effects of various concentrations of glutaraldehyde on melanocyte macromolecular synthesis as measured by incorporation of [^{3}H]-Thymidine and [^{3}H]-Leucine. The results of these experiments showed that it was reproducibly possible to abolish DNA synthesis yet preserve approximately 60% of protein synthetic capacity by treating melanoma cells with 0.002% solution of glutaraldehyde in PBS for 5 min at room temperature (Table 7).

I accordingly treated melanoma cell cultures with 0.002% glutaraldehyde, washed them extensively with PBS and blocked with 0.1M glycine in RP-10 for 1 hr at 37°C to inactivate residual glutaraldehyde molecules. I then added the test fibroblasts. As can be seen from the results summarized in Fig. 11, melanoma cells in which DNA synthesis had been abolished by glutaraldehyde treatment but in which protein synthetic capacity was reasonably intact, were still capable of inducing fibroblast collagen synthesis. Higher concentrations of glutaraldehyde, that abolished melanoma cell protein synthesis, also abolished the stimulatory effect upon fibroblasts. No inhibition of fibroblast collagen synthesis was seen, when these cells were co-cultivated with melanoma cells exposed to 0.05% glutaraldehyde.

TABLE 7

Effect of glutaraldehyde on DNA and protein synthesis by UCT-Mel 7 cells

Glutaraldehyde conc.	Incorporation of [^{3}H]-Thymidine (dpm/4 hr)	Incorporation of [^{3}H]-Leucine (dpm/4h)
0	3 913	29 018
0.002%	253	18 392
0.01%	259	523
0.05%	233	588

UCT-Mel 7 cultures ($3x10^5$/35mm dish) were treated with glutaraldehyde for 5 mins at room temperature at the indicated concentrations. After washing with RPMI, reactive aldehyde groups were blocked by incubating the cultures in 100mM glycine in RP-10 for 1 hr at 37°C. The cultures were then incubated for 24 hrs in RP-10 before being pulsed for 4 hrs with [^{3}H]-Thymidine or [^{3}H]-Leucine (5μCi/dish in each case). The cultures were then washed with PBS and the radiolabelled macromolecules precipited with TCA (5% final concentration), collected and counted.

Values represent the average of the results of duplicate dishes.

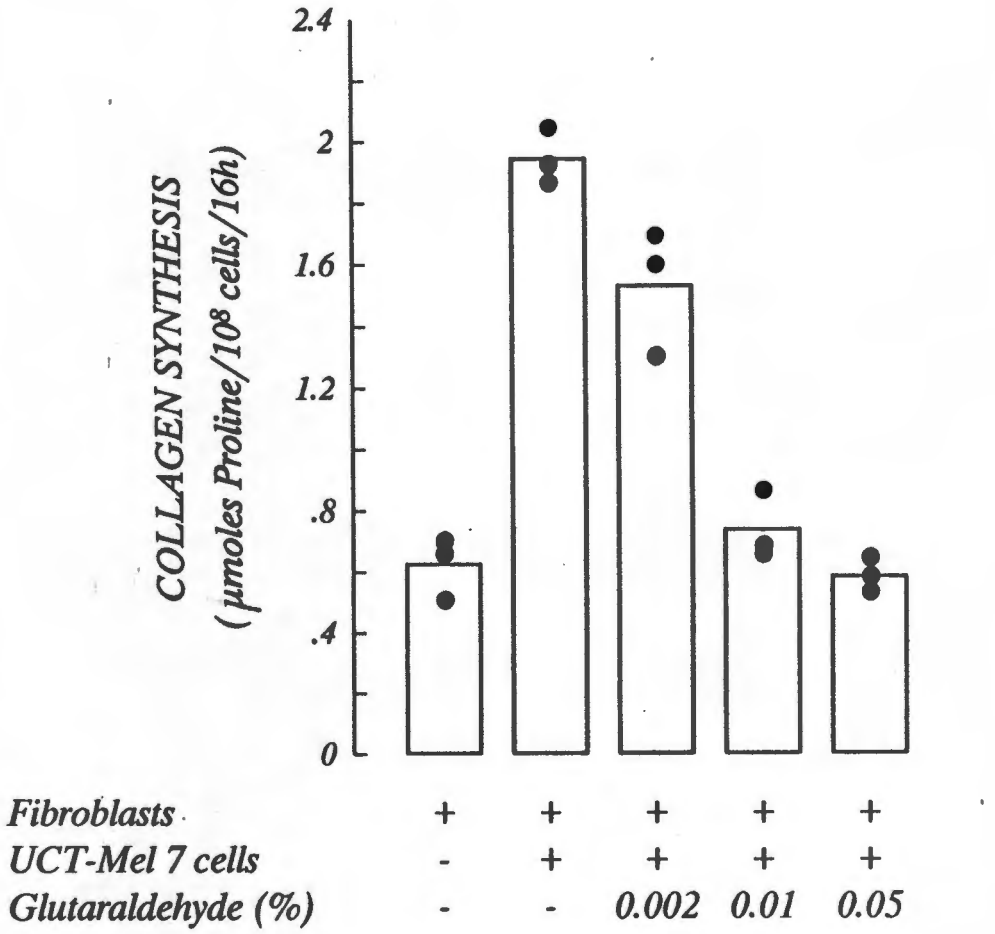


FIGURE 11

Effect of pretreatment of UCT-Mel 7 cells with glutaraldehyde on their ability to stimulate fibroblast collagen synthesis

UCT-Mel 7 cells ($3x10^5$/35mm dish) were treated with glutaraldehyde for 5 min at room temperature at concentrations ranging from 0.002% to 0.05%. After washing with RPMI, reactive aldehyde groups were blocked by incubating the cultures in 100mM glycine in RP-10 for 1 hr at 37°C. Fibroblasts ($3x10^5$/35mm dish) were then added and the cultures incubated for 24 hr before being labelled for 16 hr with 5μCi [^{3}H]-Proline for the measurement of collagen synthesis. Each point represents the values of individual dishes. Bar heights represent the average of the results of the triplicate dishes.

UCT-Mel 7 cells treated with 0.002% glutaraldehyde - a concentration that partially inhibited protein synthesis but totally blocked DNA synthesis - retained their ability to stimulate fibroblast collagen synthesis. Higher concentrations of glutaraldehyde abolished the stimulatory effect of UCT-Mel 7 cells.

Effect of retinoids, dexamethasone, PMA and indomethacin.

It has recently become clear that pharmacological modulators of cellular function may act synergistically with physiological stimuli to alter their effects in a biologically significant way. Glucocorticoids (Barouski-Miller and Gelehrter, 1982; Yoshikawa and Sabol, 1986) and tumour promoters (Pluznik and Mergenhagen, 1986; Kido et al., 1987) provide well documented examples of this phenomenon. I, thus, felt that it would be of interest to examine the consequences of adding these compounds to fibroblasts growing alone or in co-culture with melanoma cells. The results of these experiments are shown in Tables 8-11 and may be summarized as follows:

(i) Dexamethasone at 10^{-6} M consistently and significantly inhibited fibroblast collagen synthesis (Table 8). The inhibitory effect was also evident at 10^{-7} M but only reached an acceptable level of statistical significance in 1 of the 3 experiments. Of interest is the fact that at 10^{-8} M dexamethasone stimulated collagen synthesis in all 3 experiments and in all cases this was significant at the 5% level or less. With the exception of 2 dexamethasone concentrations in one experiment, no significant interaction between melanoma cells and dexamethasone was observed indicating that the hormone had no major effect upon the ability of melanoma cells to stimulate collagen synthesis.

(ii) Retinoic acid at 10^{-7} M and 10^{-8} M significantly ($p \leq 0.025$) increased fibroblast collagen synthesis; 10^{-6} M had no effect (Table 9). At all concentrations of retinoic acid, there was no significant interaction between melanoma cells and retinoic acid.

(iii) In one experiment indomethacin had no direct effect on fibroblast collagen synthesis (Table 10). At 10^{-8} M, there was a significant ($p < 0.005$) interaction between indomethacin and UCT-Mel 7 cells; the compound augmented the increased rate of collagen synthesis induced by the melanoma cells.

(iv) The phorbol ester, PMA, showed a highly significant dose-dependent inhibition of fibroblast collagen synthesis (Table 11). With the exception of one PMA concentration in one experiment, no significant interaction between melanoma cells and PMA was seen.

<u>The increase in collagen synthesis induced by melanoma cells did not appear to be an effect that was secondary to an effect on fibroblast proliferation.</u>

It is generally taken to be the rule that cellular proliferation and expression of the differentiated phenotype are inversely related. Inasmuch as collagen synthesis can be regarded as a differentiated fibroblast function, one might expect to find that

TABLE 8

TABLE 8

Stimulation of fibroblast collagen synthesis by UCT-Mel 7 cells: effect of dexamethasone[1]

Expt No.	UCT-Mel 7	Concentration of Dex (M) 0	-6	-7	-8	p[3]
1	-	1.111[2]	0.619	0.796	1.123	
	+	1.849	1.911	2.669	2.400	<0.001
p[4](Dex)			0.05	NS	0.05	
p[5](Dex x Mel 7)			0.025	NS	0.05	
2	-	1.143	0.707	1.036	1.454	
	+	1.452	0.839	1.306	1.964	<0.001
p(Dex)			<0.001	0.025	<0.001	
p(Dex x Mel 7)			NS	NS	NS	
3	-	1.02	0.702	0.832	1.132	
	+	1.638	1.285	1.571	1.821	<0.001
p(Dex)			<0.005	NS	<0.001	
p(Dex x Mel 7)			NS	NS	NS	

Footnotes to Table 8

1. Confluent fibroblast monolayers were cultured with or without added melanoma cells in RP-10 containing the different concentrations of dexamethasone given. After 24 hr, media were replaced with serum-free media containing the same concentration of added compound and 5μCi [^{3}H]-Proline. After a further 16 hr of incubation, the dishes were processed for the measurement of radioactive collagen. To establish the co-cultures, UCT-Mel 7 cells ($3x10^5$/35mm dish) were added to the confluent fibroblast monolayers at time 0.

2. Figures in the table give the number of μmoles of [^{3}H]-Proline fincorporated into collagen/10^8 fibroblasts/16 hr. Each figure is the average of the results of triplicate dishes.

3. p values under this heading reflect the level of significance associated with the melanoma cell effect.

4. p values in this row reflect the levels of signficance associated with the dexamethasone effect for each dexamethasone concentration relative to controls incubated in the absence of dexamethasone.

5. p values in this row indicate the levels of significance for the interaction between dexamethasone and melanoma cells.

p values in this table were calculated using a standard two-way analysis of variance for triplicate dishes. Details of the analysis are given in the Appendix (Tables A.1 to A.3).

TABLE 9

TABLE 9

Stimulation of fibroblast collagen synthesis by UCT-Mel 7 cells: effect of retinoic acid[1]

UCT-Mel	Concentration of Retinoic Acid(M)				
7	0	-6	-7	-8	p[3]
-	0.968[2]	0.958	1.106	1.289	
+	2.029	2.132	2.320	2.374	<0.001
p(RA)[4]		NS	0.025	0.01	
p(RA x Mel 7)[5]		NS	NS	NS	

Footnotes to Table 9

1. Confluent fibroblast monolayers were cultured with or without added melanoma cells in RP-10 containing the different concentrations of retinoic acid given. After 24 hr, media were replaced with serum-free media containing the same concentration of added compound and 5μCi [^{3}H]-Proline. After a further 16 hr of incubation, the dishes were processed for the measurement of radioactive collagen. To establish the co-cultures, UCT-Mel 7 cells ($3x10^5$/35mm dish) were added to the confluent fibroblast monolayers at time 0.

2. Figures in the table give the number of μmoles of [^{3}H]-Proline incorporated into collagen/10^8 fibroblasts/16 hr. Each figure is the average of the results of triplicate dishes.

3. p values under this heading reflect the level of significance associated with the melanoma cell effect.

4. p values in this row reflect the levels of signficance associated with the retinoic acid effect for each retinoic acid concentration relative to controls incubated in the absence of retinoic acid.

5. p values in this row indicate the levels of significance for the interaction between retinoic acid and melanoma cells.

p values in this table were calculated using a standard two way analysis of variance for triplicate dishes. Details of the analysis are given in the Appendix (Table A.4).

TABLE 10

TABLE 10

Stimulation of fibroblast collagen synthesis by UCT-Mel 7 cells: effect of indomethacin[1]

UCT-Mel 7	Concentration of Indomethacin(M) 0	-6	-7	-8	p[3]
-	0.879[2]	0.881	0,839	0.852	
+	1.644	1.699	1,758	2.069	<0.001
p(Indomethacin)[4]		NS	NS	NS	
p(Indomethacin x Mel 7)[5]		NS	NS	0.005	

Footnotes to Table 10

1. Confluent fibroblast monolayers were cultured with or without added melanoma cells in RP-10 containing the different concentrations of indomethacin given. After 24 hr, media were replaced with serum-free media containing the same concentration of added compound and 5μCi [^{3}H]-Proline. After a further 16 hr of incubation, the dishes were processed for the measurement of radioactive collagen. To establish the co-cultures, UCT-Mel 7 cells ($3x10^5$/35mm dish) were added to the confluent fibroblast monolayers at time 0.

2. Figures in the table give the number of μmoles of [^{3}H]-Proline incorporated into collagen/10^8 fibroblasts/16 hr. Each figure is the average of the results of triplicate dishes.

3. p values under this heading reflect the level of significance associated with the melanoma cell effect.

4. p values in this row reflect the levels of signficance associated with the indomethacin effect for each indomethacin concentration relative to controls incubated in the absence of indomethacin.

5. p values in this row indicate the levels of significance for the interaction between indomethacin and melanoma cells.

p values in this table were calculated using a standard two way analysis of variance for triplicate dishes. Details of the analysis are given in the Appendix (Table A.5).

TABLE 11

TABLE 11

Stimulation of fibroblast collagen synthesis by UCT-Mel 7 cells: effect of PMA[1]

Expt No	UCT-Mel 7	Concentration of PMA(ng/ml) 0	10	1	0.1	p[3]
1	-	1.152[2]	0.291	0.557	0.936	
	+	2.024	0.511	1.094	1.831	<0.001
p(PMA)[4]			<0.001	<0.001	NS	
p(PMA x Mel 7)[5]			0.025	NS	NS	
2	-	0.886	0.142	0.483	1.001	
	+	1.058	0.541	0.738	1.244	<0.001
p(PMA)			<0.001	<0.001	NS	
p(PMA x Mel 7)			NS	NS	NS	

Footnotes to Table 11

1. Confluent fibroblast monolayers were cultured with or without added melanoma cells in RP-10 containing the different concentrations of PMA given. After 24 hr, media were replaced with serum-free media containing the same concentration of added compound and 5μCi [^{3}H]-Proline. After a further 16 hr of incubation, the dishes were processed for the measurement of radioactive collagen. To establish the co-cultures, UCT-Mel 7 cells ($3x10^5$/35mm dish) were added to the confluent fibroblast monolayers at time 0.

2. Figures in the table give the number of μmoles of [^{3}H]-Proline incorporated into collagen/10^8 fibroblasts/16 hr. Each figure is the average of the results of triplicate dishes.

3. p values under this heading reflect the level of significance associated with the melanoma cell effect.

4. p values in this row reflect the levels of signficance associated with the PMA effect for each PMA concentration relative to controls incubated in the absence of PMA.

5. p values in this row indicate the levels of significance for the interaction between PMA and melanoma cells.

p values in this table were calculated using a standard two way analysis of variance for triplicate dishes. Details of the analysis are given in the Appendix (Table A.6 to A.8).

actively dividing cells synthesize less collagen than those that were quiescent. By the same argument melanoma cells, if they were to inhibit fibroblast growth, might stimulate collagen synthesis.

With this possibility in mind, I did a number of experiments designed specifically to examine the effects of co-cultivation with melanoma cells on fibroblast proliferative potential.

Fibroblast and melanoma cells were plated at a ratio of 1:1 so that each 35 mm dish contained $5x10^4$ fibroblasts. Control dishes contained an equivalent number of fibroblasts alone. At time points taken 1, 3, 4, 7 and 8 days after plating, the cells were released with trypsin and counted. In the mixed cultures fibroblasts were separated from melanoma cells by rosetting with monoclonal antibody-coated erythrocytes as described in the methods section. The melanoma cells had no effect upon the fibroblast growth rate (Fig. 12).

Inductive effects mediated by cell:cell contact.

As I have indicated in my account of the experiments performed up to this point, I was unable to detect a soluble factor that was released by melanoma cells that could be held responsible for the induction of collagen synthesis. It remains to consider other ways in which melanoma cells could have exerted their effects without the release of detectable soluble factors in the medium.

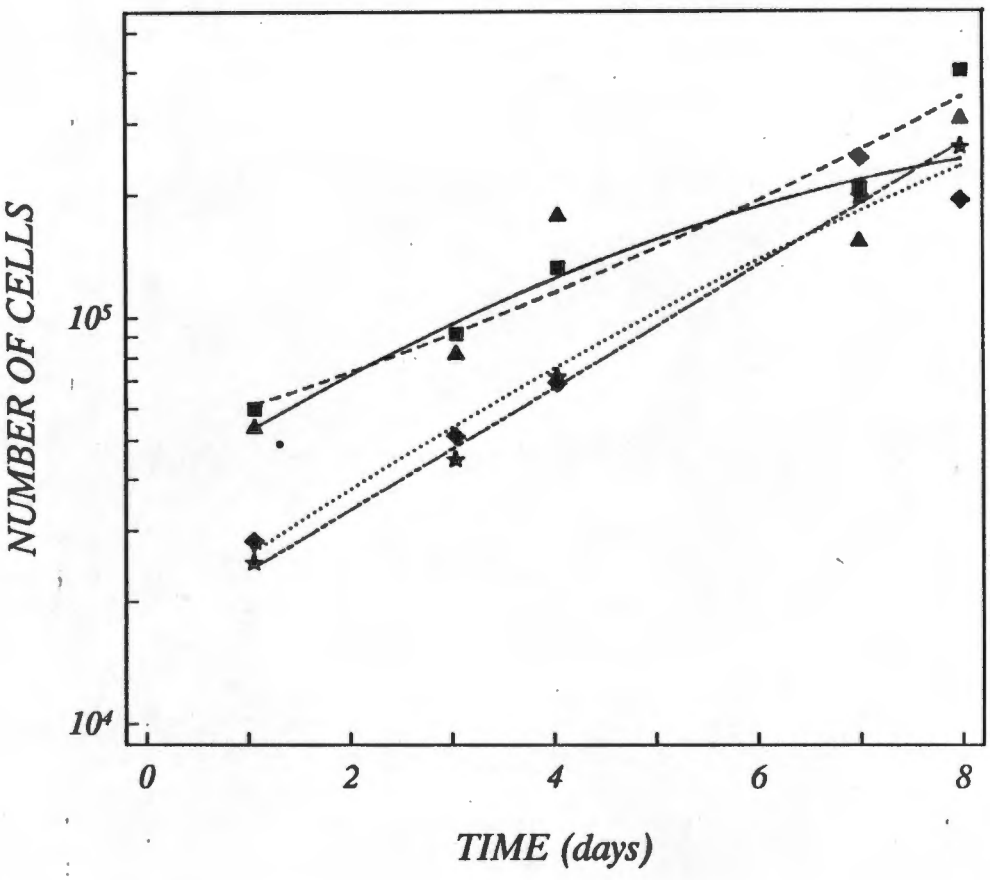


FIGURE 12

Effect of co-culture on the growth rates of UCT-Mel 7 cells and fibroblasts

Fibroblasts and UCT-Mel 7 cells were plated separately or as mixed cultures in 35mm dishes on Day 0 and the medium (1ml of RP-10) was changed on days 2, 3, 5 and 7. The cells in duplicate dishes were released with trypsin, separated using antibody-conjugated erythrocytes and counted at the indicated times.

The figure shows the growth curves of:

1. Fibroblasts, alone ▲——▲
2. Fibroblasts, co-cultured with UCT-Mel 7 cells ■----■
3. UCT-Mel 7, alone ♦·····♦
4. UCT-Mel 7, co-cultured with fibroblasts ★—·—★

Co-culture of the fibroblasts and melanoma cells had no effect on the growth rate of either of cell type.

Three broad possibilities come to mind. Firstly, it is well known that intimate contact between cells may lead to the establishment of gap junctions and cytoplasmic fusion. This process has been most strikingly demonstrated by complementation experiments involving co-cultivation of two mutant cell types each deficient in their ability to perform a different step in a metabolic process. The passage of low (ca 1000 daltons) molecular weight metabolite intermediates through the junctions reconstitutes the net synthetic process (Davidson et al., 1984). Although not strictly analogous, it is nonetheless conceivable that gap junctions established between UCT-Mel 7 cells and fibroblasts could have transported stimulatory molecules to induce collagen synthesis. Such molecules could have exerted their effects without necessarily being detected in the medium.

Secondly, it has recently become apparent from a consideration of immunological phenomena that cell types may deliver biological signals to each other through the medium of surface molecules that are integral membrane proteins. It is known, for example, that the lymphocyte T cell receptor:CD3 complex responds to MHC Class I or Class II proteins in association with antigenic fragments that are presented on the cell surface. Similarly, cellular adhesion molecules function as mediators of mutual cellular information transfer during the course of embryogenesis, morphogenesis and histogenesis (Edelman , 1986; Thiery et al., 1985). It is therefore possible that UCT-Mel 7 cells possess integral surface

structures that are recognized by fibroblasts and transduced into effective signals for collagen synthesis.

Thirdly, it is now well known that intercellular communication in a number of interacting systems is mediated by components of the ECM. Although an entirely satisfactory model to unify the biochemistry of cell:ECM interactions has yet to be developed, a number of impressive experiments (Liotta, 1986; Fransson, 1987; Le Douarin, 1984; Watt, 1986; Gospodarowicz and Greenburg, 1981) have suggested ways in which this might occur. ECM components (e.g. heparan sulphate) may bind to growth factors or biologically active molecules without compromising their activity. By sequestration, the local half-lives of the growth factors are prolonged; they are protected from proteolytic degradation; and they are concentrated on cellular surfaces or other substrata and so depleted from the surrounding fluid phase (Roberts et al., 1988; Gordon et al, 1987; Folkman et al., 1988; Vlodavsky et al., 1987; Saksela et al., 1988).

Experiments that have demonstrated these effects of ECM components have generally used a number of approaches to identify ECM:growth factor interactions. Heparin, as a close analogue of heparan sulphate, proved to be a valuable reagent for the affinity purification of fibroblast growth factor and has been shown by a number of workers to exert biological effects in cellular systems where fibroblast growth factor is active (Uhlrich et al., 1986; Sprugel et al., 1987; Roberts et al., 1988; Folkman et al.,

1988). Others have used enzymes that specifically degrade ECM components (such as hyaluronate, chondroitin sulphate, heparan sulphate, collagen etc.) to abrogate the effects of added growth factors on cellular function.

Research in haemopoietic growth factors has benefitted from experiments in which, for example, GM-CSF could be released from its bound state by extracting the ECM:growth factor complex with dissociating agents such as 2M sodium chloride, KSCN or high concentrations of arginine (Gordon et al., 1987). The biological activity of the depleted ECM could be restored by adding back growth factor and washing.

It is known that cellular behaviour may be modified by associations with ECM that are mediated by soluble macromolecules, such as fibronectin, that engage cellular receptors belonging to the recently recognized "integrin" superfamily. Evidence for interactions of this sort is usually derived from experiments in which small fragments of the binding molecules (usually oligopeptides containing the tetrapeptide sequence Arg-Gly-Asp-Ser) are shown to inhibit cellular associations or biological effects (Grabel and Watts, 1987; Pierschbacher and Ruoslahti, 1984).

Finally, during the past decade a number of biologically active peptides have been identified that have a profound effect upon the function of fibroblasts and other cell types (Gospodarowicz, 1987). These so-called "fibroblast growth factors" (FGFs) have

strong historical and experimental associations with ECM and heparin - indeed, their strong affinity for heparin was effectively used as a means for their purification - and it is well recognized that ECM may have profound effects upon the morphology and function of cells cultured in vitro. These known associations between ECM, its component proteoglycans and FGFs suggested the possibility that they might be involved in the melanoma cell:fibroblast interactions that I have observed: a suggestion that was emphasized by the apparently absolute requirement for the intimate cell:cell contact this interaction required.

With these considerations in mind, I performed a number of experiments to explore cell contact events as possible causes for the melanoma:fibroblast effects that I had seen. The results of these may be summarized as follows:

Gap junction inhibitors had no effect on melanoma cell induction of fibroblast collagen synthesis.

In a series of elegant experiments using co-cultivated fibroblasts from two patients - one with argininosuccinate synthetase (ASS)-deficiency and the other with argininosuccinate lyase (ASL)-deficiency - Davidson and his associates (1986) were able to show that α-glycyrrhetinic acid is a potent inhibitor of gap junctions. I accordingly performed the experiments in which melanoma cells and fibroblasts were co-cultured in the presence or absence of various concentrations of glycyrrhetinic acid, including those that were known to inhibit complementation of ASS^- and ASL^-

cells. Appropriate control experiments were done to ensure that the inhibitor was not toxic or inhibitory in the non-specific sense. The results showed no effect of the inhibitor (Fig. 13).

bFGF, heparin and melanoma cells acted synergistically to modulate fibroblast collagen synthesis.

To investigate the possible involvement of bFGF and ECM, I started with a series of preliminary experiments to define experimental conditions. The results of these, in summary, were as follows:

a) bFGF (2.5ng/ml - 40ng/ml) consistently inhibited fibroblast collagen synthesis and stimulated fibroblast proliferation in a dose-dependent manner. The results of a typical experiment are presented in Fig. 14.

b) Heparin was somewhat inconsistent in the magnitude of the effect that it produced. Two stock solutions were prepared, one of which (Table 12 Expt. 1) inhibited collagen synthesis at concentrations as low as 5μg/ml whereas the other (Table 12 Expt. 2) had no significant effect upon collagen synthesis, producing only minimal inhibition at 10μg/ml. In subsequent more definitive experiments, however, both preparations inhibited significantly at 10μg/ml (Table 13).

Much of the interest in the actions of FGFs and ECM lies not so much in their primary effects as in the manner in which they

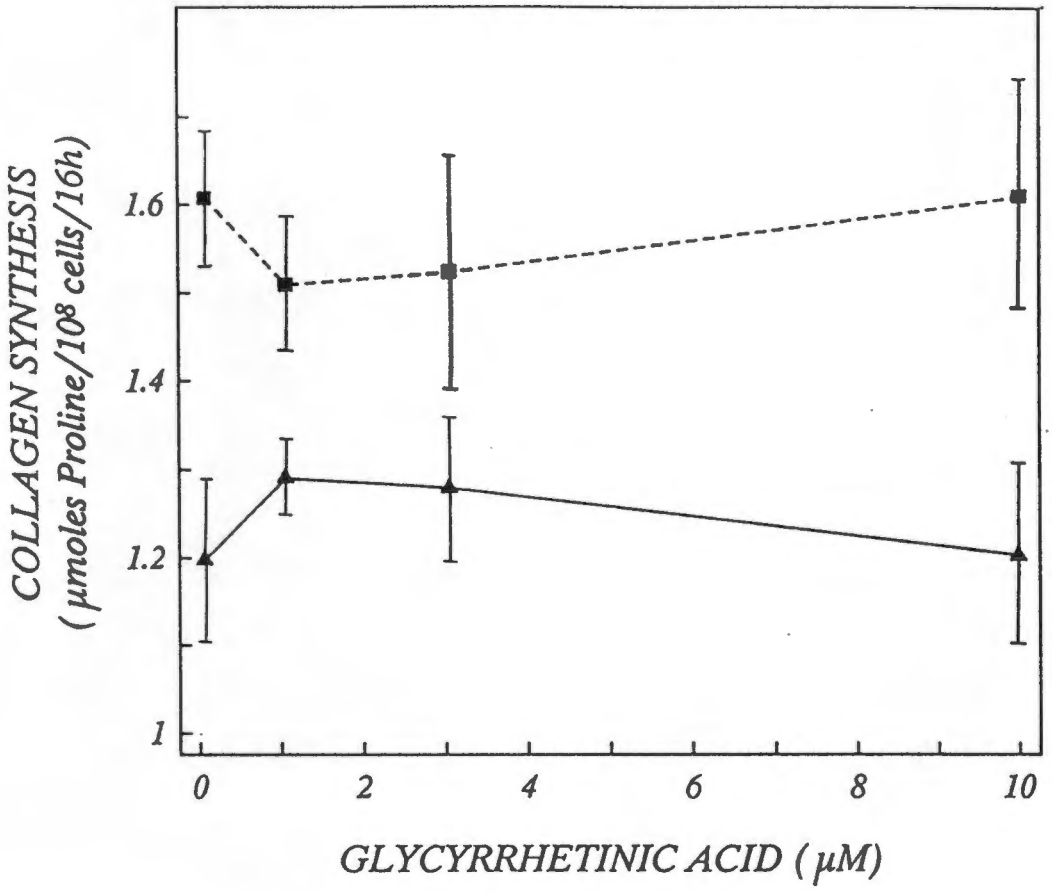


FIGURE 13

Stimulation of fibroblast collagen synthesis by UCT-Mel 7 cells: effect of the gap junction inhibitor, glycyrrhetinic acid

Confluent fibroblast monolayers were cultured either alone (▲——▲) or as co-cultures with melanoma cells (■----■) in RP-10 containing the indicated concentrations of glycyrrhetinic acid for 24 hrs. After a further 16hr of incubation in serum-free RPMI containing 5μCi [^{3}H]-Proline and the inhibitor, the dishes were processed for the measurement of collagen synthesis. To establish the co-cultures UCT-Mel 7 cells (3x10^5/35mm dish) were plated on confluent fibroblast monolayers at time 0.

Each point represents the average of the results of triplicate dishes. Error bars represent 1 s.e.m.

Glycyrrhetinic acid had no effect on the stimulation of collagen synthesis.

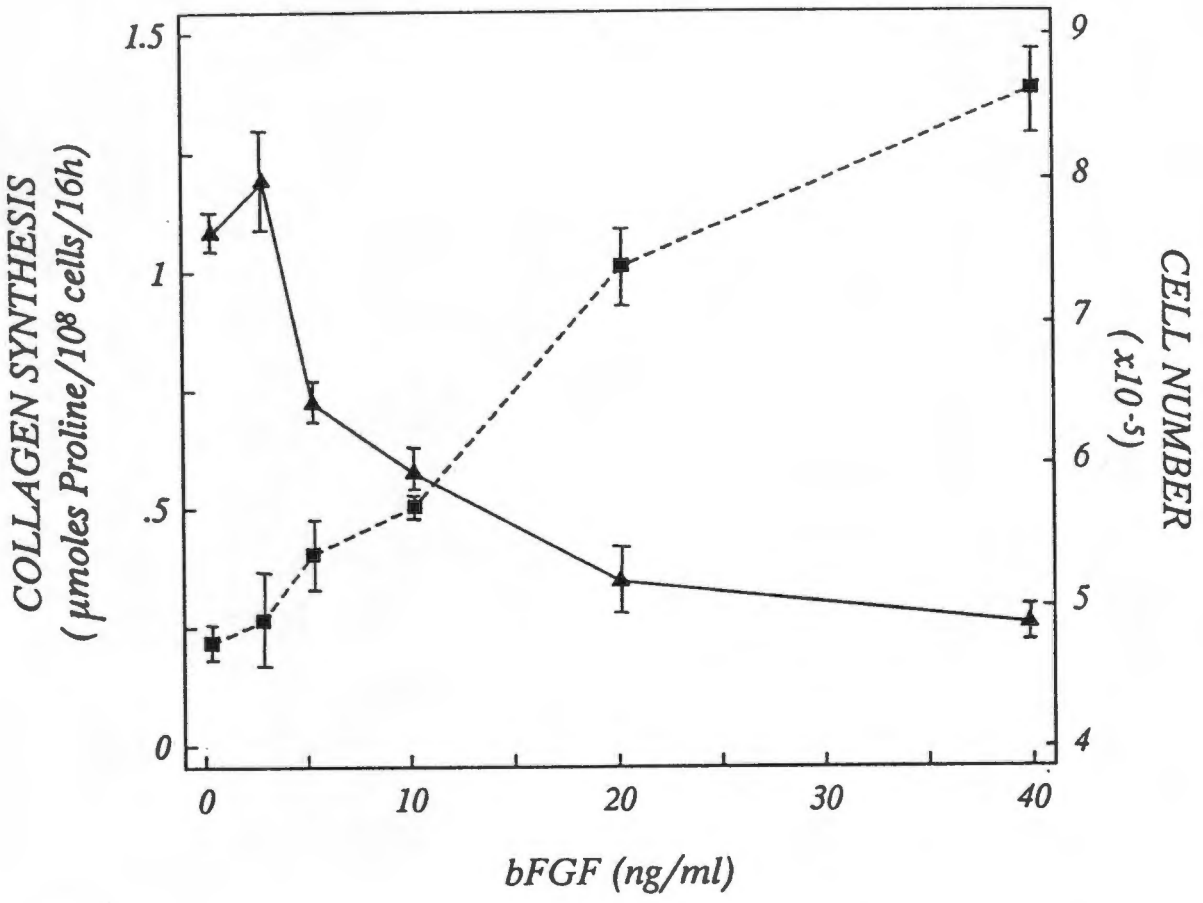


FIGURE 14

The effect of bFGF on fibroblast collagen synthesis and proliferation

The figure shows the results of a typical experiment in which confluent fibroblast monolayers were incubated with recombinant bFGF at the indicated concentrations for 24 hr. The cultures were then labelled for 16 hr with 5µCi [^{3}H]-Proline for the measurement of collagen synthesis.

Each point represents the average of the results of 3 dishes. Error bars represent 1 s.e.m.

bFGF caused a dose-dependent inhibition of fibroblast collagen synthesis (▲——▲) and stimulation of fibroblast proliferation (■----■).

TABLE 12

Effect of heparin on fibroblast collagen synthesis and proliferation

Heparin conc. (μg/ml)	Cell No. ($x10^{-5}$)	Collagen Synthesis (μmoles proline/10^8 cells/16 hr)
Expt. 1		
0	5.7	0.96
5	5.9	0.55
10	6.82	0.35
20	6.48	0.30
Expt. 2		
0	4.7	1.082
1.25	4.2	1.262
2.5	4.79	1.124
5	4.64	1.251
10	5.45	0.887
20	4.15	0.966

Confluent fibroblast monolayers were incubated for 24 hr in RP-10 containing heparin at the indicated concentrations and then for a further 16 hr in RPMI containing 5μCi [^{3}H]-Proline. The culture supernatants were processed for the measurement of radioactive collagen.

Each value represents the average of the results of triplicate cultures.

Heparin was somewhat inconsistent in the magnitude of the effect that it produced. In experiment 1, it inhibited fibroblast collagen synthesis. In experiment 2, it produced only minimal inhibition at 10μg/ml.

interact. Since it is well recognized that experiments that are designed on a factorial basis are most suitable for the detection of such interactions (Bailey, 1959; Brownlee, 1957).

I planned the following series of experiments in such a way that triplicate cultures were exposed to all possible combinations of treatments at 2 levels of each factor (i.e. UCT-Mel 7 cells, bFGF and heparin). The results are presented in detail in the Appendix and in summary in Table 13 together with p values calculated from the standard 3-way analysis of variance (Bailey, 1959; Brownlee, 1957) to indicate the significance of the primary and synergistic effects that were observed.

Although the magnitude of the effects in each of the 2 experiments differed, the results were surprisingly reproducible and allowed the same conclusions to be drawn. These may be summarized as follows:-

(i) UCT-Mel 7 cells, as previously observed, stimulated collagen synthesis.

(ii) bFGF inhibited collagen synthesis when added at 2.5 or 40 ng/ml.

(iii) heparin, when added at 10 or 20μg/ml, inhibited collagen synthesis.

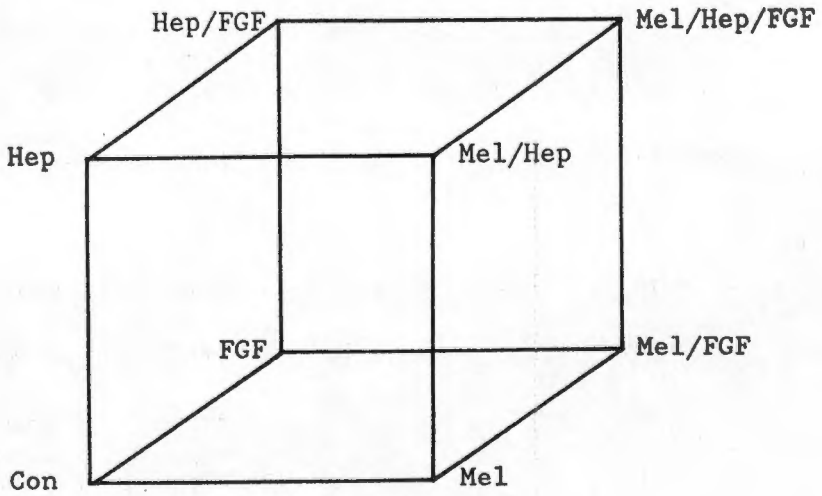
(iv) UCT-Mel 7 cells synergized with bFGF in the sense that the effect of the melanoma cells was significantly less marked in the presence of bFGF than in its absence. This interaction

TABLE 13

LEGEND TO TABLE 13

In considering the results of these "2^3" factorial experiments it is convenient to define the three "treatments" or "factors" as Mel, Hep and FGF representing UCT-Mel 7 cells, heparin and bFGF, respectively, each applied at two levels. Treatment combinations may then be depicted as Mel, Hep, FGF, Mel/Hep, Mel/FGF, Hep/FGF, Mel/Hep/FGF and Con where "Mel" signifies treatment with UCT-Mel 7 cells alone; Mel/Hep signifies treatment with UCT-Mel 7 cells in the presence of heparin etc; and Con signifies controls with no treatments applied.

The 2^3 factorial design is conveniently portrayed as a "cubic" diagram showing the treatments as points on the corners of a cube (as shown in Figure). Reference to this diagram will help to clarify the notation used and illustrates the fact that each corner of the cube is related to its
appropriate control by a single edge.



The primary effects are calculated as follows:

(i) UCT-Mel 7 cells

1/4(Mel + Mel/FGF + Mel/Hep + Mel/Hep/FGF) - 1/4(Con + FGF + Hep + Hep/FGF)

(ii) Heparin

1/4(Hep + Mel/Hep + Hep/FGF + Mel/Hep/FGF) - 1/4(Con + Mel + FGF + Mel/FGF)

(iii) bFGF

1/4(FGF + Mel/FGF + Hep/FGF + Mel/Hep/FGF) - 1/4(Con + Mel + Hep + Mel/Hep)

The interactions are calculated as follows:

(i) UCT-Mel 7 x bFGF

1/2(Con + Hep) - 1/2(Mel + Mel/Hep) - 1/2(FGF + FGF/Hep) + 1/2(Mel/FGF + Mel/FGF/Hep)

(ii) UCT-Mel 7 x Heparin

1/2(Con + FGF) - 1/2(Mel + Mel/FGF) - 1/2(Hep + Hep/FGF) + 1/2(Mel/Hep + Mel/FGF/Hep)

(iii) Heparin x bFGF

1/2(Con + Mel) - 1/2(Hep + Mel/Hep) - 1/2(FGF + Mel/FGF) + 1/2(Hep/FGF + Mel/Hep/FGF)

TABLE 13

Fibroblast collagen synthesis: effects of bFGF heparin and UCT-Mel 7 cells. Summary of experimental results[1]

Primary effects[2]	Fibroblast collagen synthesis (μmoles proline/10^6 cells/16hr)			
	0	+	Δ	p[3]
Expt. 1				
UCT-Mel 7 cells	0.395	0.600	+0.205	<0.001
Heparin(20μg/ml)	0.688	0.308	-0.381	<0.001
bFGF(40ng/ml)	0.805	0.190	-0.615	<0.001
Expt. 2				
UCT-Mel 7 cells	0.468	0.776	+0.308	<0.001
Heparin(10μg/ml)	0.804	0.440	-0.364	<0.001
bFGF(2.5ng/ml)	0.734	0.510	-0.224	<0.001

1. These tables summarize the results of factorial experiments in which triplicate dishes containing confluent fibroblast monolayers were treated with all possible combinations of melanoma cells, heparin and recombinant bFGF at the concentrations indicated. After 40 hrs of culture, during the last 16 hrs of which the cells were labelled with 5μCi [^{3}H]-Proline, the cultures were processed for the measurement of collagen synthesis. The tables were constructed from data presented in Table A.9 in the Appendix.

2. Primary effects and interactions were calculated as defined in the legend.

3. p values indicate the level of significance attributable to observed differences due to the compounds themselves.

4. p values for the interactions referred to the level of significance of the synergistic effect. p values were calculated from the 3-way analysis of variance for triplicate samples presented in the Appendix (Table A.10).

Interactions

Fibroblast collagen synthesis

(μmoles proline/10^8 cells/16 hr)

(i) UCT-Mel 7 x FGF

	Expt. 1		Expt. 2	
bFGF(ng/ml)	0	40	0	2.5
0	0.635	0.155	0.530	0.405
$3x10^5$ Mel 7 cells	0.975	0.220	0.938	0.615
Δ'	0.340	0.070	0.408	0.615
Δ''	-0.270(<p0.001)[4]		-0.198(p<0.025)	

(ii) UCT-Mel 7 x Heparin

	Expt. 1		Expt. 2	
Hep(μg/ml)	0	20	0	10
0	0.535	0.255	0.561	0.124
$3x10^5$ Mel 7 cells	0.840	0.360	1.047	0.506
Δ'	0.305	0.105	0.486	0.382
Δ''	-0.200(p<0.01)		-0.104(p<0.001)	

(iii) Heparin x bFGF

	Expt 1		Expt. 2	
bFGF(ng/ml)	0	40	0	2.5
No heparin	1.155	0.220	0.865	0.743
Heparin	0.455	0.160	0.603	0.277
Δ'	+0.700	-0.060	0.262	0.466
Δ''	+0.640 (p<0.001)		+0.204 (p<0.025)	

can be given the alternative emphasis of stating that bFGF inhibited collagen synthesis more profoundly in the presence of melanoma cells than in their absence.

(v) UCT-Mel 7 cells and heparin acted synergistically in much the same way as melanoma cells and bFGF since the inhibitory effects of heparin were more pronounced in the presence of melanoma cells or, alternatively, the melanoma cells were signficantly less stimulatory in the presence of heparin than in its absence.

(vi) As expected, heparin and bFGF showed a significant synergistic inhibitory interaction.

It is of interest to note that the magnitude of the effect induced by bFGF was sensitive to the protocol used (Table 14). When bFGF, at 2.5μg/ml, was added to confluent fibroblast monolayers that had been established in the absence of bFGF, the average inhibition of collagen synthesis (Δ = -0.245 μmoles proline/10^6 cells/16 hr) was minimal and the interaction between UCT-Mel 7 cells and bFGF not significant. Use of a different protocol, however, in which the bFGF was added, together with the fibroblasts, to established monolayers of UCT-Mel 7 cells resulted in a highly significant and pronounced inhibition of collagen synthesis with no signficant synergy.

TABLE 14

TABLE 14

Stimulation of fibroblast collagen synthesis by UCT-Mel 7 cells: effect of recombinant bFGF[1]

Additions	Collagen synthesis[4] (μmoles proline/10^8 cells/16 hr)			Average
Expt. 1.[2]				
(a) None	0.564	0.637	0.485	0.562
(b) bFGF	0.467	0.583	0.628	0.559
(c) UCT-Mel 7	1.045	1.138	1.320	1.168
(d) bFGF + UCT-Mel 7	0.969	0.810	0.998	0.926

i) Effect of bFGF (b + d) - (a + c) = -0.245 (NS)[5]

ii) Effect of UCT-Mel 7 (c + d) - (a + b) = 0.973 ($p < 0.001$)

iii) Interaction (a - b) - (c - d) = -0.239 (NS)

Expt. 2[3]				
(a) None	1.464	1.702	1.538	1.568
(b) bFGF	0.558	0.429	0.528	0.505
(c) UCT-Mel 7	2.547	2.214	2.537	2.432
(d) bFGF + UCT-Mel 7	1.573	1.318	1.550	1.480

i) Effect of bFGF (b + d) - (a + c) = -2.015 ($p < 0.001$)

ii) Effect of UCT-Mel 7 (c + d) - (a + b) = 1.839 ($p < 0.001$)

iii) Interaction (a - b) - (c - d) = 0.111 (NS)

Footnotes to Table 14

1. These two experiments, both of which were designed to examine the effect of bFGF on melanoma cell stimulation of collagen synthesis, are presented to demonstrate the sensitivity of the experimental system to the protocol that is used. In experiment 1, melanoma cells and bFGF are added to confluent fibroblast monolayers that had been established in the absence of bFGF. In experiment 2, it was the melanoma cells that were established cultures and fibroblasts and bFGF were added to these monolayers.

2. Confluent fibroblast monolayers (± $6x10^5$/35mm dish) were washed and covered with 1 ml of RP-10 containing $3x10^5$ UCT-Mel 7 cells and 2.5ng bFGF in the various combinations shown. The cultures were incubated for 24 hrs followed by 16 hrs in serum-free RPMI containing 5μCi [^{3}H]-Proline.

3. Semi-confluent monolayers of UCT-Mel 7 cells (± $5x10^5$/35mm dish) were covered with 1 ml RP-10 containing $3x10^5$ fibroblasts and 2.5ng of bFGF in the various combinations shown. Thereafter the protocol was the same as for experiment 1.

4. Values in the body of the table give the rate of collagen synthesis (μmoles proline/10^8 cells/16 hr) measured in each individual dish and the averages.

5. p values indicate the significance of the
(i) effect of bFGF
(ii) effect of melanoma cells
(iii) interaction between bFGF and melanoma cells

p values were obtained from F-test ratios in a standard two-way analysis of variance presented in the Appendix (Table A.11).

Matrix deposited by melanoma cells had no effect on fibroblast collagen synthesis.

In the event that the melanoma cells were depositing a matrix on the plastic surface of the tissue culture dish that was responsible for inducing fibroblast collagen synthesis, I performed the experiment in which the melanoma cells were plated and allowed to grow to semi-confluence at which stage they were removed from the dish using procedures that are known to leave behind deposited ECM. After gentle washing of the dishes, they were used to plate fibroblasts for the measurement of collagen synthesis using the standard protocol. No effects of the matrix could be discerned (Fig. 15).

Dialysed extracts of cells, cell membranes and conditioned matrix components did not show stimulatory activity.

A number of reports have appeared to indicate that growth factors bound non-covalently to ECM may be released by extraction under dissociating conditions. I accordingly performed a number of experiments in which melanoma cells were treated in such a way as to favour the release of bound growth factors. The extracts were dialysed to restore a physiological composition and added to fibroblasts in the hope that this would detect stimulatory factors. None were found. An extract prepared with Tris buffered sodium deoxycholate was inhibitory (Fig. 16).

Lyophilised acetic acid extracts of melanoma cell cultures and preparations of melanoma cell membranes were similarly ineffective.

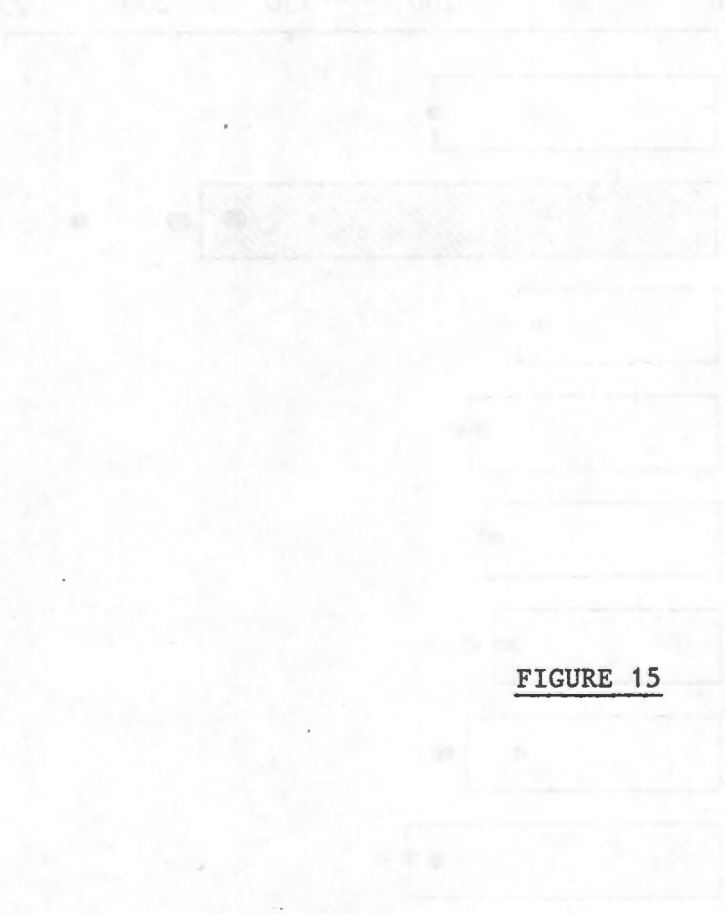


FIGURE 15

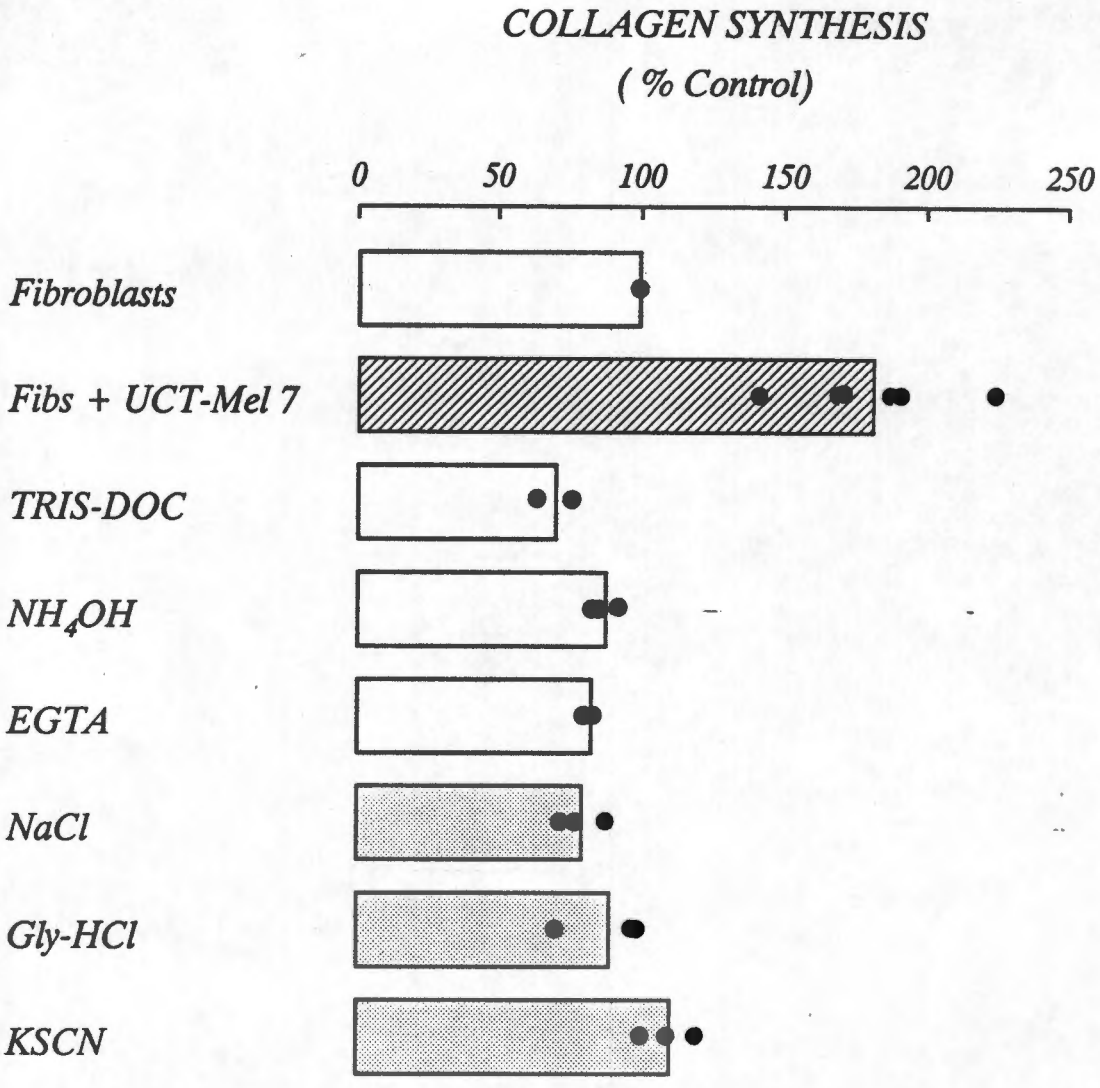

COLLAGEN SYNTHESIS
(% Control)
0
50
100
150
200
250
Fibroblasts
Fibs + UCT-Mel 7
TRIS-DOC
NH4OH
EGTA
NaCl
Gly-HCl
KSCN

FIGURE 15

FIGURE 15

Fibroblast collagen synthesis: effect of UCT-Mel 7 cells and UCT-Mel 7 derived matrix

Fibroblasts were plated at a confluent density on melanoma cells ($3x10^5$/35 mm dish) or on matrix preparations prepared from UCT-Mel 7 cells according to the following protocols. Literature references are given in parentheses.

(i) Cell-free extracellular matrix (Clear bars)

a) Tris-DOC (Laiho et al., 1986) - cells were extracted 3 x 5min with 10mM Tris-HCl pH 8 + 0.5% Na deoxycholate at room temperature. The residue on the plate was washed thoroughly and the fibroblasts added.

b) NH_4OH (Kao et al., 1984) - cells were removed from the dish by treatment with 25mM NH_4OH for 2 minutes at room temperature. The extracted matrix was washed and the fibroblasts added.

c) EGTA (Biswas, 1985) - cells were released with 0.5mM EGTA at 37°C. The matrix was washed and the fibroblasts added.

(ii) Cell-associated matrix (stippled bars)

Cell-associated matrix was prepared by extracting UCT-Mel 7 monolayers for 30 min at room temperature with:

a) 2M NaCl in water (Gordon et al., 1987)

b) 1.6M KSCN in water

c) 0.1M glycine HCl pH 2.8.

The residue remaining on the dish was washed with PBS, air-dried overnight and the fibroblasts plated on to the dishes the next day.

Cultures were then incubated for 24 hrs in RP-10, following by 16 hr in serum-free RPMI containing 5μCi [^{3}H]-Proline. The supernatants were then processed for the measurement of radiolabelled collagen.

Points and bar heights depict, respectively, results of individual dishes and averages expressed as a percentage of the average values for dishes in which fibroblasts were cultured alone.

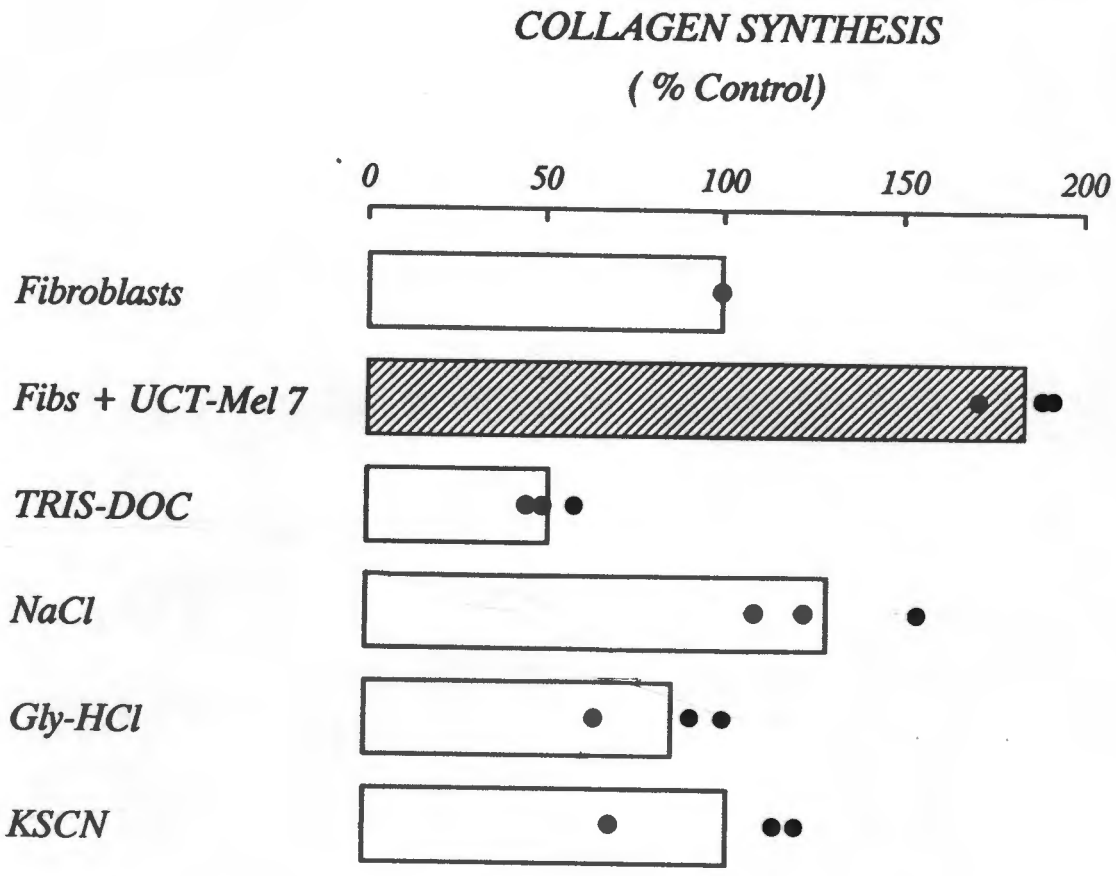


FIGURE 16

Extracts of matrix deposited by melanoma cells: effect on fibroblast collagen synthesis

In this experiment, performed to complement the experiment described in Fig. 15, material extracted from the cells according to the protocols described in the legend to Fig. 15 were dialysed (Spectra/Por 4 membrane tubing; versus RPMI for 16 hrs at 4°C), sterile filtered (0.45μm Millex HA) and added to confluent fibroblast monolayers.

Each dish contained approximately $4x10^5$ fibroblasts and received $5x10^5$ cell equivalents of extracted cellular material. The cultures were incubated for 40 hrs during the last 16 hrs of which 5μCi [^{3}H]-Proline was present in each dish. The dishes were then processed for the measurement of radioactive collagen.

Each point represents the value for an individual dish normalized to a value of 100% for the cultures in which fibroblasts were incubated alone. Bar heights represent the average.

An extract of melanoma cells prepared with a mixture of HCl and ethanol, according to techniques that are commonly used for the isolation of TGF-β (Roberts et al., 1984), showed signficant inhibition of collagen synthesis (Fig. 17).

<u>The effects of melanoma cell on fibroblast collagen synthesis are exerted at a transcriptional or post-transcriptional level.</u>

The synthesis of Type I collagen involves a complex series of sequential steps, most of which lend themselves to regulatory influences (Reviewed in Literature Review). For the sake of discussion and experimental design, I have found it convenient to consider processes that regulate the rate of collagen synthesis as those which operate to regulate the amount of mRNA that is available for translation and secondly, those that affect translation of the mRNA and assembly and secretion of the final product. If the inductive effects of co-culture with melanoma cells operated at the first of these two levels - i.e. by increasing transcription or by stabilization of the mRNA - one would expect to find increased levels of collagen mRNA in the cells after contact with melanoma cells. If, on the other hand, melanoma cells influenced only the rate of translation of available mRNA or the activities of synthetic enzymes involved in the post-translational processing, assembly and secretion of collagen, one would expect to find similar amounts of collagen mRNA in co-cultivated and control fibroblasts.

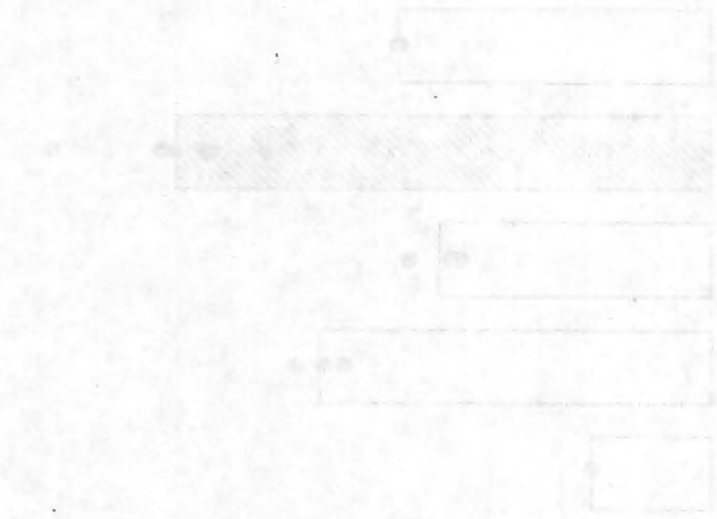


FIGURE 17

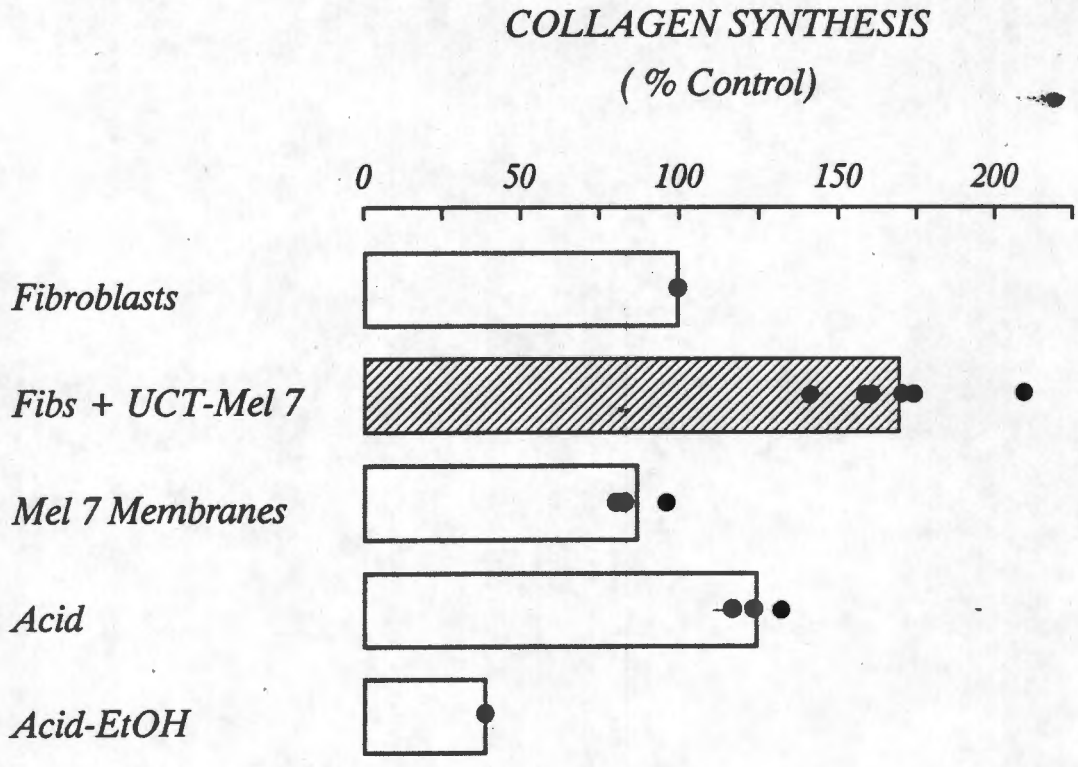

COLLAGEN SYNTHESIS
(% Control)
0
50
100
150
200
Fibroblasts
Fibs + UCT-Mel 7
Mel 7 Membranes
Acid
Acid-EtOH

FIGURE 17

FIGURE 17

Fibroblast collagen synthesis: effects of melanoma cells or melanoma cell derived membranes or extracts

In this experiment, confluent fibroblast monolayers were treated with melanoma cells ($3x10^5$/35mm dish) or with membranes or extracts prepared from melanoma cells according to the following protocols. Literature references are given in parentheses.

a) Membranes. A crude membrane preparation was obtained using the method of Nara and McCulloch (1985) in which the cells were lysed by hypotonic lysis (10mM KCl, 1.5mM $MgCl_2$, 2mM PMSF, 10mM Tris HCl pH 7.4), disrupted in a Polytron homogenizer and centrifuged at 400g to remove nuclei and 100 000g to pellet the membranes. The pellet, equivalent to $6x10^5$ cells/ml, was resuspended in RPMI and added to the fibroblasts.

b) Acid extraction. UCT-Mel 7 cells were extracted with 2M acetic acid + 1mM PMSF for 16 hr at 4°C. After centrifugation at 12 000g, the supernatant was lyophilized. The lyophilized extract was reconstituted in water and re-lyophilized three times. The extract was then reconstituted in RPMI to give $2x10^6$ cell equivalents/ml and added to the fibroblasts.

c) Acid-Ethanol (Roberts et al., 1984). UCT-Mel 7 cells were extracted with hydrochloric acid:ethanol buffer for 2 hrs at room temperature after disruption in a Polytron homogenizer. The extract was centrifuged at 12 000g and the supernatant precipitated overnight with a mixture of ether and ethanol. The precipitate was collected on filter paper, dissolved in 2M acetic acid and lyophilized as for the acid extraction. The extract was then reconstituted in RPMI to given $2x10^6$ cell equivalents/ml and added to the fibroblasts.

The cultures were incubated for 40 hrs during the last 16 hrs of which 5μCi [^{3}H]-Proline was present in each dish. The dishes were then processed for the measurement of radiolabelled collagen.

Each point represents the value for an individual dish normalized to a value of 100% for the cultures in which fibroblasts were incubated alone. Bar heights represent the average.

To resolve this issue I performed an experiment in which fibroblasts were cultured either alone or with melanoma cells and processed to determine the amount of collagen mRNA they contained. The experiment involved separating fibroblasts and melanoma cells after cultivation: this was achieved by the use of erythrocyte-linked monoclonal antibodies and centrifugation on a step density gradient as described in the Methods section. Fibroblasts cultured alone were mock separated using the same procedure.

Total cellular RNA was isolated from each of the two fibroblast populations and analysed by electrophoresis and Northern blotting using nick-translated α1(I) and α2(I) collagen gene probes. As a reference standard that would allow assessment of the specificity of the inductive effect, I compared the signal for collagen mRNA to that obtained when Northern blots were probed with nick-translated actin DNA. The results (Fig. 18) showed quite clearly that co-cultivation with melanoma cells resulted in a selective increase of collagen mRNA relative to actin mRNA.

At any given time, the amounts of collagen mRNA could be expected to reflect net processes of transcription and degradation. Had the melanoma cell effect resulted in increased mRNA levels by induction of increased transcription, one would have predicted that the transcriptional inhibitor, α-amanitin, would, when used at a concentration that inhibited RNA synthesis, abrogate the melanoma cell effect. This prediction was tested in an experiment in which

I co-cultivated fibroblasts and melanoma cells in the presence or absence of 5μg/ml α-amanitin.

The results of this experiment are shown in Fig. 19. α-amanitin inhibited collagen synthesis by the fibroblasts and totally abrogated the inductive effect of UCT-Mel 7.

Inhibitory effects of bFGF and heparin are associated with diminution of intracellular collagen mRNA.

To determine the regulatory level at which bFGF and heparin were operating, I incubated fibroblasts with the compounds, either alone or in combination, isolated total cellular RNA and analysed this by electrophoresis and Northern blotting using nick-translated α1(I) and α2(I) collagen gene probes. The results (Fig. 20) showed quite clearly that heparin had negligible effects on intracellular mRNA; bFGF alone decreased the amount of collagen mRNA; and heparin and bFGF in combination appreciably decreased the amount of collagen mRNA. Heparin and bFGF, either alone or in combination, had no effect on β-actin mRNA.

Inhibition of proteoglycan synthesis abrogated the melanoma cell induction of fibroblast collagen synthesis

Cell surface proteoglcans have been shown to be important in a number of cell:cell interactions. The proliferation and development of haemopoietic stem cells occurs in close association with bone marrow stromal cells (Roberts et al., 1988). Similarly, Schwann cell proliferation is stimulated by co-culture with dorsal root

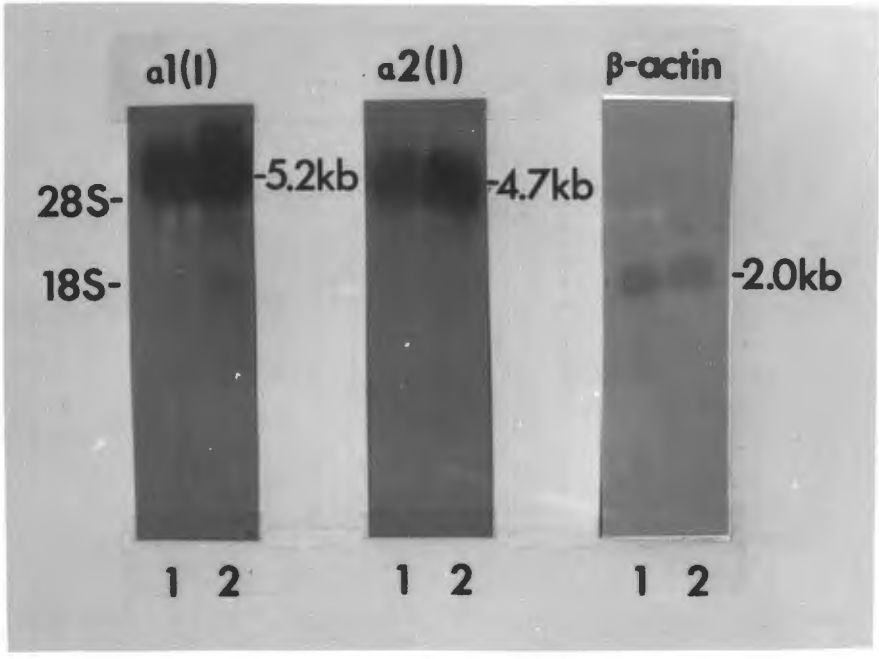


FIGURE 18

Effect of UCT-Mel 7 cells on fibroblast collagen mRNA

Confluent fibroblast monolayers (approximately 10^7 cells/100mm dish) were cultured alone or as co-cultures with $8x10^6$ UCT-Mel 7 cells for 48 hrs. The cells were then released with trypsin, incubated with erythrocytes coated with anti-melanoma cell antibody and centrifuged on a Lymphoprep cushion. Fibroblasts separated from melanoma cells or mock-separated were collected from the Lymphoprep:medium interface and used to prepare total cellular RNA. The RNA (20μg/lane) was electrophoresed on a 1.5% denaturing formaldehyde-agarose gel, blotted on to Hybond N paper and hybridized with nick-translated [^{32}P]-labelled probes specific for α1(1) collagen, α2(1) collagen and β-actin.

In each autoradiograph, lane 1 contains RNA from fibroblasts cultured alone and lane 2 contains RNA from fibroblasts that had been co-cultured with UCT-Mel 7 cells.

Co-cultured fibroblasts contained more α1(1) and α2(1) collagen mRNA than fibroblasts cultured alone; co-culture had no effect on β-actin mRNA.

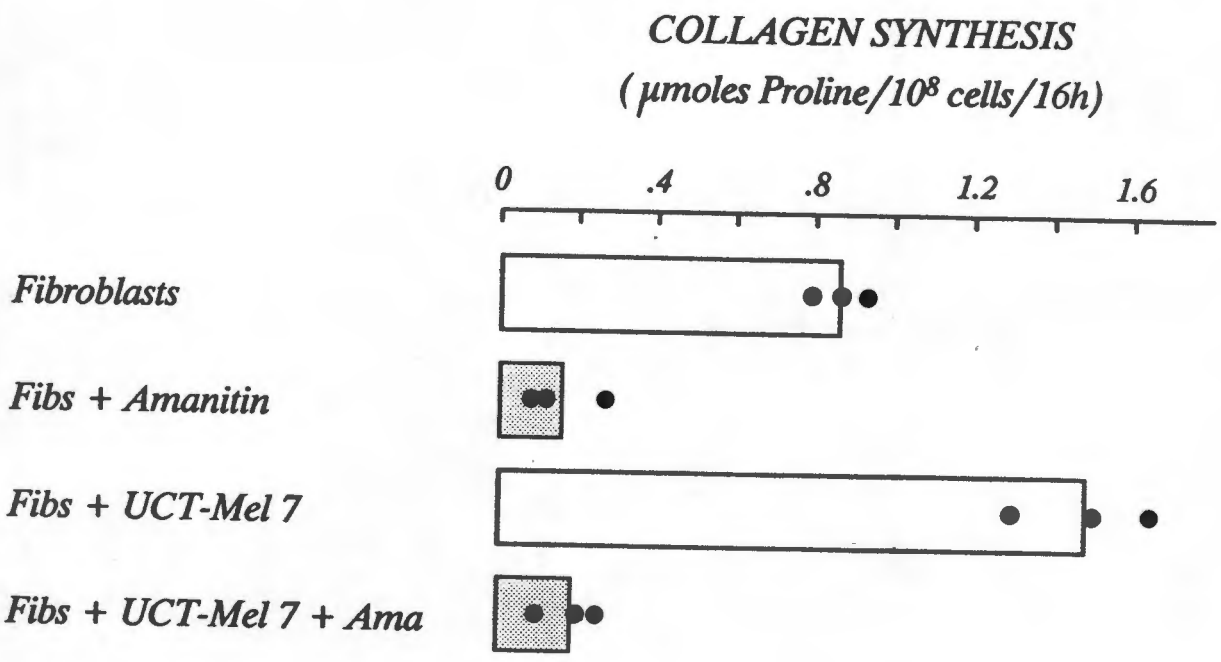


FIGURE 19

Stimulation of fibroblast collagen synthesis by UCT-Mel 7 cells: effect of α-amanitin, a specific inhibitor of mRNA synthesis

Fibroblasts or fibroblast:UCT-Mel 7 co-cultures were incubated for 40 hrs with or without 5μg/ml of α-amanitin. For the last 16 hrs of culture, 5μCi [^{3}H]-Proline was added. Cultures were then processed for the measurement of radioactive collagen.

Confluent fibroblast monolayers were established in 35mm dishes; UCT-Mel 7 cells ($3x10^5$/dish) and α-amanitin were added to experimental dishes at time zero. Points and bar heights represent, respectively, values for individual dishes and average values.

α-amanitin inhibited collagen synthesis by the fibroblasts and totally abrogated the inductive effect of UCT-Mel 7 cells.

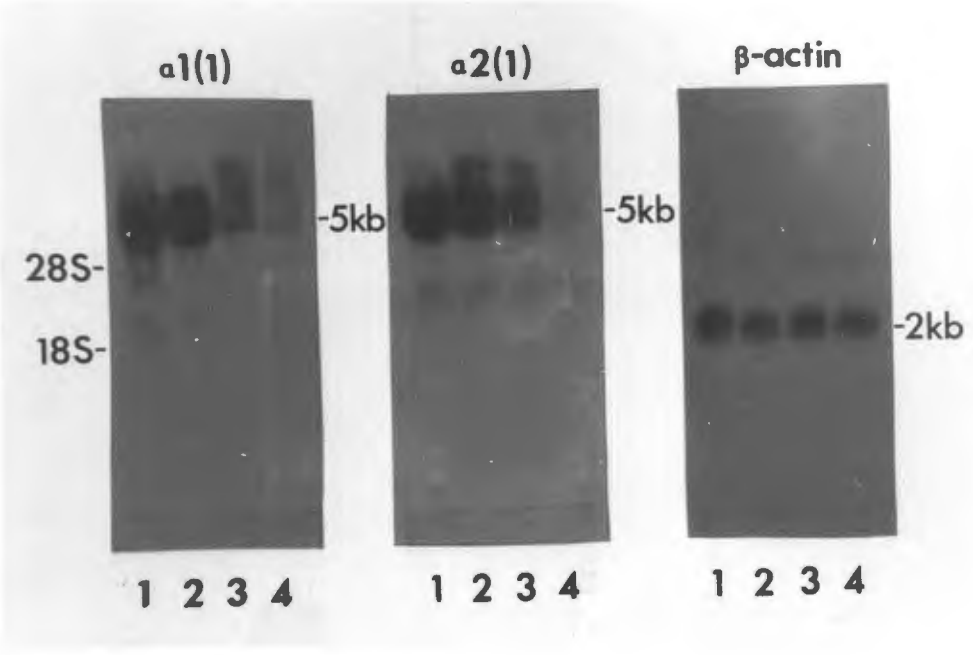


FIGURE 20

Effect of bFGF and heparin on the accumulation of type I collagen and β-actin mRNA's in cultured fibroblasts

Confluent fibroblast monolayers were treated for 48 hrs with bFGF (40ng/ml), heparin (20μg/ml) or a combination of both compounds. The compounds were not added to control dishes. Total cellular RNA was extracted and 20μg RNA/lane was electrophoresed on a 1.5% denaturing formaldehyde-agarose gel. The separated RNA was blotted on to Hybond-N paper and hybridized to [^{32}P]-labelled nick-translated DNA probes specific for α1(1) collagen, α2(1) collagen or β-actin.

Lane 1 contained RNA from control fibroblasts.
Lanes 2 - 4 contained RNA from fibroblasts treated with heparin (lane 2), bFGF (lane 3), heparin together with bFGF (lane 4).

The autoradiographs showed that heparin had negligible effects on intracellular collagen mRNA; bFGF alone decreased the amount of collagen mRNA; and heparin and bFGF in combination acted synergistically to decrease the amount of collagen mRNA. Heparin and bFGF, either alone or in combination, had no effect on the amount of β-actin mRNA.

ganglion neurons (Ratner et al., 1985). Co-culture of melanoma cells with fibroblasts, induces the fibroblasts to synthesize increase amounts of collagenase. In all cases, cell surface proteoglycans play a role and have been identified as heparan sulphate. I therefore decided to look for proteoglycan involvement in the interactions between fibroblasts and melanoma cells that I had seen.

Normal biosynthesis of xylose-linked proteoglycans (i.e. chondroitin sulphates, dermatan sulphates, heparins and heparan sulphates) can be inhibited by the addition of exogenous β-D-xylosides (Ratner et al., 1985; Thompson and Spooner, 1983). These compounds compete with the xylosated core protein at the level of the first galactosyltransferase. This leads to an increase in free glycosaminoglycan chains in the medium and inhibits glycosaminoglycan addition to proteoglycan core proteins (Galligani et al., 1975; Lohmander and Hascall, 1979; Thompson and Spooner, 1983). I, therefore, studied the effects of 4-methylumbelliferyl-β-D-xyloside (β-D-xyloside) on melanoma cell induction of fibroblast collagen synthesis.

In all, three experiments have been performed to study the effects of β-D-xyloside on fibroblast collagen synthesis and the fibroblast:melanoma cell interaction. In the first experiment (Table 15,Expt 1) confluent fibroblast monolayers established in the absence of any added compounds were incubated alone or with UCT-Mel 7 cells and β-D-xyloside in DMSO (1mM xyloside in 1% DMSO,

final concentrations) in all combinations. The results showed highly significant primary effects of UCT-Mel 7 cells and β-D-xyloside: UCT-Mel 7 cells stimulated fibroblast collagen synthesis and β-D-xyloside inhibited fibroblast collagen synthesis. There was also a significant inhibitory interaction between the melanoma cells and β-D-xyloside ($p < 0.025$). In other words, the effect of the melanoma cells was less marked in the presence of β-D-xyloside than in its absence.

In the second experiment (Table 15,Expt 2) fibroblasts and β-D-xyloside in all combinations were added to monolayers of UCT-Mel 7 cells established in the absence of added compounds. Once again the primary effects of UCT-Mel 7 cells and β-D-xyloside were highly significant ($p < 0.001$) but, in this experiment, there was no significant synergy between the melanoma cells and β-D-xyloside. The different results obtained in these 2 experiments, as with bFGF, highlights the sensitivity of the experimental system to the protocol used.

I felt that the experimental design used in these 2 experiments was not really adequate for completely stripping the cell surface of its proteoglycans. The addition of β-D-xyloside to established cultures would have stopped synthesis of new proteoglycans but need not have depleted the peri-cellular matrix to a significant extent if the turnover was slow. For this reason, I decided to pretreat the fibroblasts with β-D-xyloside for 72hrs before adding the melanoma cells. The results of this experiment are shown in Fig. 21.

TABLE 15

Footnotes to Table 15

1. These two experiments are presented to demonstrate the effect of β-D-xyloside on fibroblast collagen synthesis and the fibroblast melanoma cell interaction.
In experiment 1, melanoma cells and β-D-xyloside were added to confluent fibroblast monolayers that had been established in the absence of β-D-xyloside.
In experiment 2, it was the melanoma cells that were established cultures and fibroblasts and β-D-xyloside were added to these monolayers.

2. Confluent fibroblast monolayers (± $7x10^5$/35mm dish) were covered with 1 ml RP-10 containing $3x10^5$ UCT-Mel 7 cells and 1mM 4-methylumbelliferyl-β-D-xyloside (β-D-xyloside) in the various combinations shown. The cultures were incubated for 24 hr, followed by 16 hr in serum-free RPMI containing 5μCi [^{3}H]-Proline.

3. Monolayers of UCT-Mel 7 cells (± $5x10^5$/35mm dish) were covered with 1 ml RP-10 containing $3x10^5$ fibroblasts and 1mM β-D-xyloside in the various combinations shown. Thereafter the protocol was the same as for Experiment 1.

4. Values in the body of the table give the rate of collagen synthesis (μmoles proline/10^8 cells/16 hr) measured in each individual dish and the averages.

5. p values indicate the significance of the
i) effect of β-D-xyloside
ii) effect of melanoma cells
iii) interaction between β-D-xyloside and melanoma cells

p values were obtained from F-test ratios in a standard two-way analysis of variance presented in the Appendix (Table A.12).

FIGURE 21

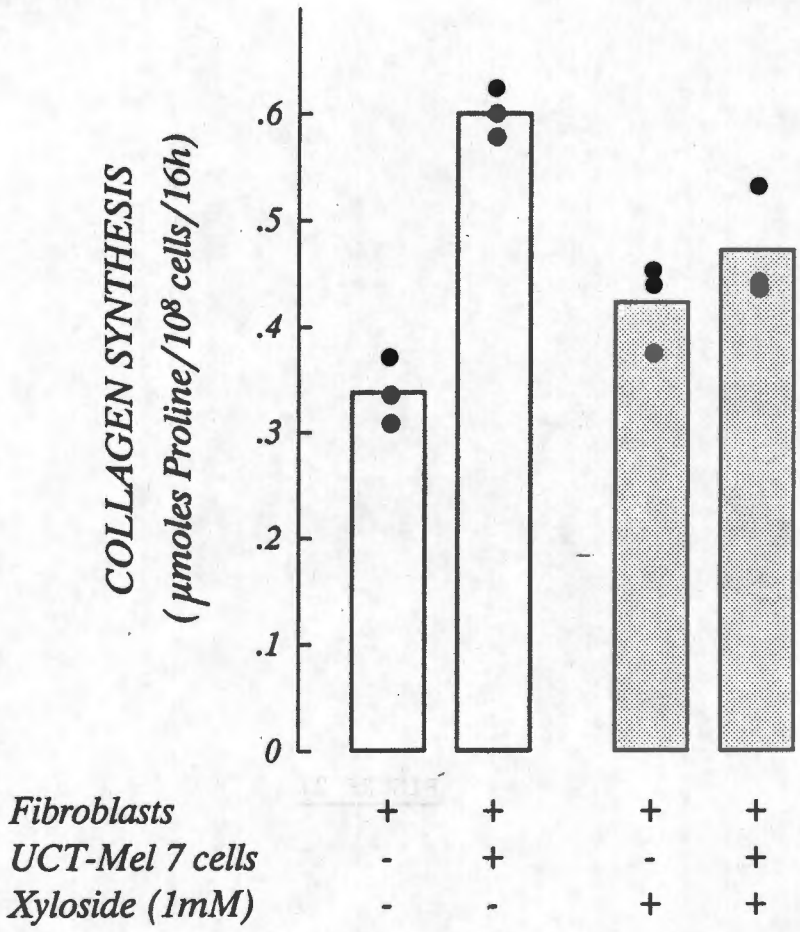

COLLAGEN SYNTHESIS
(µmoles Proline/10⁸ cells/16h)
.6
.5
.4
.3
.2
.1
0
Fibroblasts + + + +
UCT-Mel 7 cells - + - +
Xyloside (1mM) - - + +

FIGURE 21

FIGURE 21

Stimulation of fibroblast collagen synthesis by UCT-Mel 7 cells: effect of 4-methylumbelliferyl-β-D-xyloside

Fibroblasts were plated at $1x10^5$ cells/35mm dish in 2ml DB-10. Twenty microliters of a 100mM solution of β-D-xyloside in DMSO was added to experimental dishes; control dishes received DMSO alone. The dishes were incubated for 72 hrs with daily medium changes until the fibroblasts were confluent. UCT-Mel 7 cells ($3x10^5$) were then added to the fibroblast monolayers in RP-10 containing DMSO with or without the inhibitor and the cells incubated for a further 24 hrs during which the melanoma cells were seen to attach to the fibroblasts with a small number of floating cells. The medium was then replaced with serum-free RPMI containing DMSO with or without the inhibitor and 5μCi [^{3}H]-Proline. Radioactive collagen was measured after 16 hrs.

Bar heights represent averages of the individual points shown for each dish.

The relatively low rate of collagen synthesis seen in this experiment is attributable to the large number of fibroblasts in the dish (see Fig. 3).

Note that the melanoma cell effect was abolished by pre-incubation of the fibroblasts with the inhibitor of proteoglycan assembly.

TABLE 16

Proteoglycan synthesis by fibroblasts and melanoma cells

Cells		Incorporation of $[^{35}S]$-SO_4 (dpm/35mm dish)
Fibroblasts	Medium	82 488
	Cells	4 148
UCT-Mel-7	Medium	71 426
	Cells	18 048

Confluent monolayers of fibroblasts and UCT-Mel 7 cells were labelled with 100μCi $[^{35}S]$-SO_4 (New England Nuclear NEX 041; 10-1000mCi/mmol) for 20 hrs in 1 ml serum-free RPMI. The medium was collected and the cells were washed 3x with cold PBS and scraped from the dish into 200μl of extraction buffer (0.5% Triton X-100, 0.1% SDS, 1 mM PMSF in PBS). Cells were extracted for 30 mins at 4°C, centrifuged and the supernatant collected. Aliquots of the medium and the cell extracts were precipitated with TCA (10% final conc),centrifuged and the precipitate washed once with 10% TCA. The final precipitate was dissolved in 2N NaOH and counted.

Fibroblasts, plated at low density and grown to confluency in the presence of β-D-xyloside, failed to respond to the melanoma cells. The analysis of variance of these results showed a significant primary effect of UCT-Mel 7 cells, no significant primary effect of β-D-xyloside and a significant ($p < 0.005$) interaction between UCT-Mel 7 cells and β-D-xyloside. In other words, the melanoma cell effect was less marked in the presence of β-D-xyloside than in its absence.

Unfortunately I could not complete the symmetrical experiment in which the melanoma cells were pretreated with β-D-xyloside because the β-D-xyloside proved toxic for the melanoma cells. When plated at low density in the presence of β-D-xyloside, the melanoma cells failed to adhere and proliferate.

Both fibroblasts and UCT-Mel 7 cells synthesized proteoglycans as assessed by incorporation of [^{35}S]-sulphate into TCA-precipitable material (Table 16). A major portion of the sulphated macromolecules were found in the medium (95% for fibroblasts and 80% for UCT-Mel 7 cells).

DISCUSSION

In this chapter I present data that show, quite clearly, that UCT-Mel 7 cells induced collagen synthesis in co-cultured fibroblasts. Although the magnitude of this *in vitro* melanoma cell effect varied from experiment to experiment, it was consistently stimulatory. At UCT-Mel 7:fibroblast ratios of 1:1, fibroblast collagen synthesis rates were, on average, 80% higher than those seen in control cultures (Fig. 1).

The effect was relatively specific for collagen synthesis (since the incorporation of [^{3}H]-leucine was not affected; Fig. 7) and was dose-dependent over a range of melanoma:fibroblast ratios from 1:50 to 2:1 (Fig. 4). The induction of collagen synthesis was evident after 4 hours of co-culture (Fig. 5). Analysis of the kinetics of the effect indicated that melanoma cells increased the maximal rate of proline incorporation into collagen rather than the apparent "Michaelis constant" of the reaction. Although, in a complex multi-step system such as this, one cannot legitimately draw conclusions from experimental approaches designed to study relatively simple reactions between enzyme and substrate, I take these results to indicate that the melanoma cells acted primarily to increase the total capacity for collagen synthesis by recruiting fibroblasts to the synthesis of this protein or by diverting a greater fraction of the protein synthetic apparatus for this purpose. It seems that the melanoma cell effect was not attributable to an increased rate of proline transport into

the cells or to a change in the rate constants of the intracellular reactions involved in collagen synthesis.

This interpretation is consistent with the results of the experiments showing that co-culture with UCT-Mel 7 cells consistently increased intracellular levels of mRNA for α1(1) and α2(1) collagen. The specificity of this induction was established by showing no effect on the induction of β-actin mRNA. It is not possible to say, at present, whether the increase in intracellular collagen mRNA was due to the induction of transcription of new message or to the stabilisation of existing mRNA. This question will be resolved by the results of appropriate pulse-chase experiments.

The effects of compounds such as TGF-β (Ignotz et al., 1987; Raghow et al., 1987) or interferon (Rosenbloom et al., 1984; Stephenson et al., 1985) on fibroblast collagen mRNA have been well described, but I have found no references to the effects of cellular co-cultivation on collagen mRNA.

Fibroblasts have an <u>in vitro</u> doubling time of approximately 24 hours so that the period of culture that was required to demonstrate induction of collagen synthesis (± 40 hours) was sufficiently long for this effect to have been secondary to stimulation of fibroblast proliferation. This trivial explanation for the melanoma cell effect was excluded by experiments in which melanoma cells and fibroblasts were separated after co-culture for independent counting (Fig.12). The use of erythrocyte-linked monoclonal antibodies to melanoma and

fibroblast surface epitopes provided an effective means of achieving this separation by rosetting and density gradient centrifugation. Natali et al., (1983), Mills et al., (1983) and Wilhelm et al., (1986) have also used this useful technique to resolve mixed cell populations.

Although UCT-Mel 7 cells proved most effective at inducing fibroblast collagen synthesis, this was not an exclusive property of melanoma cells in general or of these cells in particular. Most cell types (e.g. breast carcinoma cells) reacted similarly in this regard and the "Bowes" melanoma cell line (RPMI 7272) had no effect. The basis for this heterogeneity in inductive capacity is as yet obscure. It is still an issue to be resolved since comparisons between inducing and non-inducing cells might well shed light upon the mechanism for the induction.

I have been unable to find reports in the literature of attempts to understand the desmoplastic response other than those that I have summarised in Table 17. These, for the most parts, are not strictly relevant to the results that I have obtained since few authors have addressed the question of tumour effects upon the economy of collagen. Notable exceptions are provided by the experiments reported by Naito et al., (1984) and those of Biswas (1982,1985,1987,1988).

Naito et al. co-cultured gastric carcinoma cells and fibroblasts and found that collagen synthesis was stimulated. Unlike the results that I obtained however, they found that gastric carcinoma cell conditioned medium contained a soluble fibrogenic factor that could be

demonstrated in their technique of "parabiotic co-culture" in which adjacent flasks carrying the different cell types were connected by a joining tube that incorporated a semi-permeable membrane. They, however, relied heavily on histochemical appraisal for collagen synthesis rather than upon quantitative biochemical assays. Such quantitative data as they did obtain showed similar effects (an increase in hydroxyproline content) to those that I observed.

My observations are, to some extent, inconsistent with those of Biswas and her colleagues who found that B16 melanoma cells possess a surface heparin-binding protein that interacts with extracellular matrix to induce the release of a factor that increases the synthesis of fibroblast collagenase. Had a similar phenomenon occurred in my experiments I would have expected a net <u>decrease</u> in the amount of collagen released. Furthermore, I found that fibroblasts released undetectable amounts of collagenase and that the rate of release of this enzyme was not affected by melanoma cells.

The differences between the elegant experimental results that Biswas has reported and those that I have observed may well be attributable to species differences (the B16 line is a murine line) and to differences in the experimental design. She has found that co-culture of mouse fibroblasts and mouse melanoma cells does not lead to collagenase induction: xenogeneic cultures are required. My experiments have mostly involved allogeneic human cultures.

TABLE 17: MODULATION OF MATRIX COMPONENTS BY TUMOUR CELLS

Stimulatory Cell Type	Synthetic Cell Type	Product	Effect	Soluble factor involved	References
Gastric ca cells KATO-III MKN-45 MKN-28	Human skin fibroblasts	Collagen	Co-culture stimulated production of argyrophilic collagenous fibres	Yes	Naito et al., 1984
Lung ca LX-1 Pancreatic ca DAN Melanoma TRIG	Human skin fibroblasts	Hyaluronate	Co-culture stimulated production of hyaluronate as measured by incorporation of [^{3}H]-acetate into hyaluronate	No	Knudson et al., 1984
Melanomas MM-96 FME Colon Ca HCT-8 HT-29 Breast Ca MCF-7 T-47D	Human skin fibroblasts	Glycosamino-glycans	Co-culture stimulated production of glycosaminoglycans MM-96 + HT-29 most effective HCT-8 effective FME moderately effective HA/CS ratio increased	Yes	Merrilees and Findlay, 1985.
Mouse B-16 melanoma	Rabbit synovial fibroblasts	Collagenase Type I	Co-culture stimulated production of collagenase		Biswas, 1982
			Release of stimulatory factor into medium was stimulated by matrix deposited by fibroblasts.	Sometimes 12/20 expts	Biswas, 1985
			Release of the collagenase-stimulatory factor from the plasma membrane of melanoma cells is stimulated by interaction between heparan sulphate in fibroblast matix and heparan sulphate-binding proteins in plasma membrane of tumour cells.		Biswas + Toole, 1987 Biswas, 1988.

If one shifts the emphasis from collagen to other elements of the fibroblast-secreted matrix, one finds a number of reports that document an effect of malignant cells on the synthesis of these extracellular molecules. These reports I have summarised in Table 17 from which it is evident that, in most cases, where effects have been observed they have involved stimulation rather than suppression. Furthermore, it is well known that malignant cells are capable of releasing collagenase and other proteases that are capable of degrading extracellular matrix and this has been inculpated in mediating local invasion or metastatic spread. Apart from the experiments of Biswas that I have referred to above, I am unaware of any reports that have dealt specifically with the question of effects of co-culture on the release of such proteolytic enzymes.

There seems to be no hard and fast rule regarding the existence of soluble factors that are capable of modulating the deposition of extracellular matrix. Merrilees and Findlay (1985), for example, showed that co-culture of fibroblasts with a variety of tumours led to the release of a soluble factor that stimulated the deposition of glycosaminoglycans. On the other hand, Knudson et al. (1984) who performed virtually identical experiments confirmed the stimulation, by tumour cells, of glycosaminoglycan synthesis but were unable to demonstrate a soluble factor that mediated this effect. It should also be noted that Biswas although consistently able to demonstrate the induction of collagenase by co-culture was not always able to demonstrate the presence of a soluble factor in the conditioned medium that was responsbile for mediating this effect: in one paper

(Biswas,1985) she records the fact that only 60% of the melanoma cultures contained, in the conditioned medium, the inducing molecule.

In summary therefore, the results of these descriptive experiments that I have presented confirm those of other workers and extend their observations by quantitating certain kinetic aspects of the phenomenon; by defining such biological features as species specificity and induced collagen type; and by showing that the effect of melanoma cells was relatively specific on collagen synthesis. The induction of increased cellular levels of collagen mRNA by co-culture has similarly not been reported previously.

A question arises regarding the relevance of my *in vitro* experimental results to the desmoplastic response that I observed *in vivo*. It is true that the cell line that provoked maximum desmoplasia *in vivo* (UCT-Mel 7; Chap.1 Fig.9) was most effective at inducing fibroblast collagen synthesis *in vitro* (Fig.9) and that the magnitude of the *in vitro* inductive effects was, taking into account the slow turnover rate of collagen, quantitatively sufficient to lead to the long term accumulation of excessive collagen in tumours in the mice. On the other hand, UCT-Mel 1 and UCT-Mel 2 also had an effect *in vitro* which, although less than that shown by UCT-Mel 7, was nevertheless of sufficient magnitude to have made me expect a greater *in vivo* effect than that was seen.

A second inconsistency between UCT-Mel 7 and its effects on collagen synthesis *in vivo* and *in vitro* is to be found in the fact that early

(8'), middle (20 - 30') and late (>50') passages of UCT-Mel 7 cells were equally effective as inducers of collagen synthesis *in vitro* yet it was only cells derived from middle passage cultures that gave rise to desmoplastic tumours *in vivo*.

I have no convincing explanations to offer for these inconsistencies nor do I have any satisfactory speculations to offer. The *in vivo* situation is clearly too complex to warrant confident extrapolation from *in vitro* findings and further experiments are necessary if a useful answer is to be obtained. It will be of interest, for example, to isolate clones of the middle passage cells in the hope that they may differ in their ability to induce fibrogenesis *in vivo* and collagen synthesis *in vitro*, since this might indicate the *in vivo* need for an accessory cell to complement the cells that act only *in vitro*. It would be particularly interesting if clones could be isolated that were synergistic in this respect. The availability of such cell types would mean that DNA cloning procedures with subtractive hybridization might be applied to the identification of the genes involved.

It is usually desirable in studies of this sort to note whether or not a particular cellular function or effect requires macromolecular synthesis. Unfortunately, I was unable to use conventional inhibitors of polypeptide synthesis to see if the melanoma cells required intact protein synthetic machinery in order to induce collagen synthesis. There were two reasons for my inability to do so, both of which relate to the washing of the melanoma cells cultures after exposure to the

inhibitor to remove inhibitor from the second phase of the experiment when UCT-Mel 7 cells and fibroblasts were co-cultured. In the first place, cycloheximide is a reversible inhibitor and washing would have restored melanoma cell protein synthesis so invalidating the experiment. In the second place, the concentrations of actinomycin D required to inhibit [^{3}H]-Uridine incorporation into UCT-Mel 7 cells were associated with unavoidable transfer of inhibitor into the second, co-culture phase of the experiment so that fibroblast function was inhibited totally.

I thus decided to explore glutaraldehyde as a possibly useful inhibitor in this context for the reason that the functional integrity of many biological macromolecules and structures may be selectively preserved or destroyed by exposure to glutaraldehyde at the appropriate concentration. The use of glutaraldehyde as a coupling agent for affinity chromatography (Kristiansen, 1974) and as a means for attaching functional enzymes to solid phase supports (Weetall,1976) testify to the usefulness of this compound provided optimal conditions for the particular experimental circumstances are established by careful titration. By adding a low concentration (0.002%) of glutaraldehyde to the medium I was able to inhibit DNA synthesis with only a modest reduction in the rate of protein synthesis; at higher concentrations of glutaraldehyde both protein and DNA synthesis were inhibited (Table 7).

With this system I was able to show that the melanoma cells did not require the capacity for DNA synthesis to induce fibroblast collagen

synthesis (Fig. 11) - a conclusion that was confirmed by the findings with mitomycin C (Table 5). Concentrations of glutaraldehyde that inhibited protein synthesis, however, nullified the effect of UCT-Mel 7 cells on the fibroblasts (Fig.11). Glutaraldehyde treated melanoma cells did not diminish baseline collagen synthesis nor did they appear to have any other adverse effects, indicating that there was no "spill-over" of this inhibitor into the second phase of the experiment.

To the best of my knowledge glutaraldehyde, while commonly used as a fixative or coupling reagent, has not been used as an inhibitor in in vitro cell culture experiments so that this approach is without precedent. Unfortunately the effects of glutaraldehyde are too non-specific to conclude, definitively, that melanoma cell protein synthesis is required for fibroblast induction but the findings are consistent with this interpretation. I know of no other reports to demonstrate that protein synthesis in the inducing cells is necessary for the induction of macromolecular synthesis in responding cells.

Collagen synthesis is a prominent feature of the chronic inflammatory fibrotic response and it was thus of interest to note the effect, in my system, of adding agents that are experimentally and pharmacologically associated with the induction or modulation of inflammation. Phorbol myristate acetate, retinoic acid, indomethacin and dexamethasone are examples of compounds that act in this way.

For the most part my results agreed with those of published reports. It is well known that dexamethasone and PMA inhibit collagen synthesis (Flaherty and Chojkier, 1986; Weiner et al., 1987) and my results confirmed this. Retinoic acid, however, which is said to inhibit collagen synthesis (Daly and Weston, 1978; Hein et al., 1984; Ohta and Uitto, 1987) was significantly stimulatory in my experiments. I have no explanation for this discrepancy. Although all three of these compounds had significant primary effects on fibroblast collagen synthesis, none showed any significant synergy with the melanoma cells (Tables 8-11).

It is also well known the prostaglandins, by increasing intracellular cAMP concentrations, have an inhibitory effect on collagen synthesis (Baum et al., 1978; Rennard et al., 1982). Indomethacin, by inhibiting the action of prostaglandins, blocks their effect on collagen synthesis (Clark et al., 1983; Laato and Heino, 1988). I have shown that, whilst indomethacin had no effect on fibroblast collagen synthesis by itself, it significantly augmented the increased rate of collagen synthesis induced by the melanoma cells. These results may be of importance for the understanding of the manner in which the stimulus delivered by the melanoma cells is transduced, by the fibroblast, into an effective trigger to increase the rate of collagen synthesis. It is of incidental interest to note that prostaglandins are also involved in the modulation of lymphocyte responses to antibodies presented by macrophages - another situation where intimate cell:cell contact provides the inductive stimulus (Snyder et al.,1982).

The general rule that many cell-cell interactions are mediated by paracrine effects led to the obvious suggestion that the effect of melanoma cells on fibroblasts was mediated by a soluble polypeptide or other "growth factor" released into the co-culture medium. I was, however, consistently unable to demonstrate the existence of any such compound (Table 2); the effect could only be demonstrated by co-culture of melanoma cells and fibroblasts.

Most polypeptide growth factors or mediators have molecular weights of approximately 20kD and any such compound should have been retained by the Amicon 10kD cut-off filter that I used to concentrate melanoma cells conditioned medium. The fact that harvest fluid concentrated in this way had no effect on fibroblast collagen synthesis argues against low concentration of such a growth factor as a reason for my failure to demonstrate its presence.

Since it was possible that the UCT-Mel 7 cells released labile growth factors that did not withstand harvesting or storage, I performed the Cooper dish experiments in which monolayers of UCT-Mel 7 cells and fibroblasts were separated by no more than 4mm of contiguous medium. This, too, gave no indication of a soluble mediator of the melanoma cell effect (Table 3).

Reports in the literature of soluble factors that mediate cellular interactions leading to increased deposition of collagen or extracellular matrix have been inconsistent (Table 17). Naito et al., (1984) and Merilees and Findlay (1985) provided evidence to indicate

such factors do exist; Knudson et al., (1984), like myself, were unable to demonstrate them. It is of interest to note, in the light of these observations and the experimental results that I present below, that, in at least two instances, fibroblasts that had failed to respond to conditioned medium responded to a membrane fraction prepared from the inducing cells (Biswas and Toole, 1987).

It was also conceivable that, as in the case of the experiments reported by Biswas (1985), that the melanoma cells would only release their stimulatory soluble factor in response to a stimulus from co-cultivated fibroblasts, and this may have explained my inability to detect such a factor in medium harvested from melanoma cells cultivated alone. I therefore studied the effects of conditoned medium taken from co-cultures and I found that this, as in the other cases, was devoid of stimulatory activity.

My consistent inability to demonstrate a soluble factor that was resonsible for the melanoma cell effect, in the face of the equally consistent observation that co-cultivation with melanoma cells increased the rate of fibroblast collagen synthesis, led me to the obvious conclusion that the fibroblasts received their inductive stimulus from contact with some solid-phase structure. The subsequent experiments that I performed were designed to explore the possible nature of such a stimulus and the mode of its delivery.

Three well described mechanisms served as a basis for my experimental approach: cells may associate to form gap junctions (Loewenstein,

1981; MacDonald, 1985) through which cytoplasmic contents may be exchanged; they may interact by way of complementary cell surface molecules (in much the same way as antigen-presenting cells proffer processed antigen in association with major histocompatability gene products to receptors on responsive T lymphocytes); or they may attach to elements of the extracellular matrix that function as stimulatory molecules - either alone or as structures that present growth factors or other regulatory peptides.

Gap junctions are structures that are visible under the electron microscope (Loewenstein, 1981) and are functionally demonstrable by complementation experiments such as those so elegantly performed by Davidson et al. (1984, 1986). These workers showed unequivocally that α-glycyrrhetinic acid is a potent and specific inhibitor of cytoplasmic transfer through intercellular gap junctions. I have used the lack of effect of this compound upon melanoma:fibroblast interactions as strong evidence against these being mediated by gap junctions (Fig.13).

The identification of functional cell surface molecules requires reagents that bind to the receptor or the cell surface agonist (such as monoclonal antibodies or other ligands); or cloned genes that can be shown, by transfection or similar experiments, to code for the structures in question. Unfortunately the monoclonal antibodies that I raised to melanoma and fibroblast cell surface epitopes had no effect on the cellular interactions that concerned me and I have not as yet completed the work that needs to be done with mutant cells lines or cloned genetic material. I am, therefore, unable to comment

definitively on the nature of cell surface molecules that are functionally involved in the phenomenon that I have described.

During the past decade the notion of the extracellular (ECM) or peri-cellular matrix as a regulator of cell function has gained acceptance and one now sees this heterogeneous assembly of diverse macromolecules as an important component of tissues in vivo and experimental systems in vitro. Thanks largely to the contributions of workers such as Folkman et al., 1988; Gospodarowicz, 1987; Moscatelli and Rifkin, 1988, it is now clear that the ECM may influence cellular behaviour in any of several ways.

Firstly, ECM components such as fibronectin, laminin or vitronectin, in association with the "integrin" family of cell surface receptors (Hynes, 1987), provide substrata for adherence, directed motility, tissue structure and cellular morphology.

Secondly, the ECM may associate with growth factors or other regulatory molecules to present them more efficiently to cellular receptors (Roberts et al., 1988) to localise them to a particular cellular environment (Vlodavsky et al., 1987) or to protect them from proteolytic degradation (Saksela et al., 1988). This function is well demonstrated by studies of Roberts et al. (1988), who showed that peri-cellular matrix on bone marrow stromal cells was essential for the effective presentation of GM-CSF to haemopoietic progenitor cells and by the work of Saksela et al. (1988) who showed that heparin-bound

bFGF is resistant to hydrolysis by proteases normally present in cell medium.

Finally, ECM components may bind directly to receptors present on cell surfaces to deliver proliferative stimuli - as reported, for example, by Ratner et al. (1985) who showed that heparan sulphate proteoglycans associated with dorsal root ganglion neurons, deliver mitogenic stimuli to Schwann cells - or other inductive stimuli exemplified by the case of the melanoma cell heparin-binding protein described by Biswas (1988).

The recent history of ECM research is closely associated with angiogenesis and angiogenic factor (now fibroblast growth factor) - initially for the fact that heparin affinity chromatography provided an effective means for the purification of FGF and, more recently, for the synergistic interactions between bFGF and ECM that have been so well documented by Folkman et al., 1988; Konkle and Ginsburg, 1988; Vlodavsky et al., 1987.

With this knowledge in hand I performed a series of experiments designed to explore the role of the ECM in the melanoma cell:fibroblast interactions. The results of these experiments warrant comment in the following respects:

(a) Firstly, attempts to stimulate fibroblast collagen synthesis with melanoma cell membranes, extracts of melanoma cells or ECM deposited by melanoma cells were unsuccessful. In retrospect,

these experiments suffered from a number of deficiencies that will have to be rectified when they are repeated: I did not, for example, establish definitively that the UCT-Mel 7 cells did deposit a matrix on the dish or that the methods I used to remove the cells left a residual matrix; nor did I treat such matrix as was deposited with melanoma cell conditioned medium to see if a soluble factor was present that could synergize with matrix components. I did, however, use established procedures that are recommended in the literature and that should have detected obvious direct effects of matrix components.

(b) Direct effects of heparin were inconsistent in the sense that the compound either had no effect on fibroblast collagen synthesis or it suppressed this function (Table 12). On no occasion did heparin stimulate. These results would argue against the existence of a fibroblast receptor for a melanoma cell proteoglycan of the heparan sulphate-type as a mediator of these effects.

(c) Heparin consistently inhibited the effect of the melanoma cells on fibroblast collagen synthesis (Table 13). These results strongly suggest participation of a heparan sulphate type proteoglycan in the melanoma cell:fibroblast interaction, but the nature of this participation is as yet obscure. Taken in conjunction with those summarised under (b) above, my findings are consistent with the view that the cell-associated ECM is important, in which case free

heparin would act a competitive inhibitor when added to co-culture but it would have little or no effect when added alone.

(d) In two experiments (Table 15 and Fig. 21) β-D-xyloside, a potent inhibitor of proteoglycan assembly, had a direct inhibitory effect on fibroblast collagen synthesis. If, for the sake of discussion, one disregards the possibility that this inhibitor acted upon some other cellular function, one might infer that some measure of proteoglycan synthesis is required for basal collagen synthesis to proceed in unstimulated fibroblast cultures. I have not taken further experimental steps to test this inference. Clearly this should be done for the light that it might shed upon normal fibroblast metabolism. In a third experiment, β-D-xyloside had no direct effect upon collagen synthesis (Table 15).

(e) In two experiments (Fig. 21 and Table 15) β-D-xyloside inhibited the UCT-Mel 7 effect to a greater extent than could be explained by its direct effect upon fibroblast collagen synthesis alone. These results implied that proteoglycan synthesis was required for the melanoma cell effect to be seen. In a third experiment (Table 15), however, the interaction between melanoma cells and β-D-xyloside was not significant.

As indicated above, direct and synergistic effects of β-D-xyloside were inconsistent - a fact that I attribute to the sensitivity of the assay system to the experimental protocol that I used. It proved impossible, for example, to trypsinise the UCT-Mel 7 cells and to

replate them in the presence of β-D-xyloside. If this was done the cells failed to attach to the dish and died. As a result I was not able to add fibroblasts to proteoglycan-depleted UCT-Mel 7 cells that had proliferated in the presence of the inhibitor. Similarly, the direct effects of β-D-xyloside on fibroblast collagen synthesis were not seen when the cells were plated at low density and grew to confluence in the presence of an inhibitor; they were only observed when the compound was added to established monolayers or to trypsinised cells that were replated at high density and tested within 48 hours. The resolution of the apparently conflicting results that I obtained therefore will require optimization of the protocol and careful titration of the inhibitor and other factors.

These results, although as yet somewhat inconclusive, indicate that proteoglycans are involved in the melanoma cell:fibroblast interaction and suggest the following tentative model: a cell-associated proteoglycan - possibly heparan sulphate - on either the fibroblasts or UCT-Mel 7 cells engages a proteoglycan "receptor" on the other cell type. This interaction between proteoglycan and its receptor triggers the fibroblast to synthesize increased amounts of collagen. I have shown that both melanoma cells and fibroblasts are capable of synthesizing cell-associated and secreted sulphated macromolecules (Table 16) - an essential pre-requisite for such a model. It is, by analogy with GM-CSF or bFGF and their interaction with proteoglycan, also conceivable that a melanoma cell-derived soluble factor is involved but is only capable of delivering an effective stimulus if

presented to a fibroblast receptor in association with a melanoma cell-surface proteoglycan.

This model, and by extension that of the soluble factor, are consistent with my findings and are amenable to experimental verification. Experiments designed to investigate the effect of heparanase treatment of the cells; to look for the existence of proteoglycan-binding proteins on the surface of the cells; and to characterize the proteoglycans produced by the two cell types are called for.

This model is not without precedent in the literature. Biswas and Toole (1987) have shown that cell-associated proteoglycans play a role in the induction of increased amounts of Type I collagenase in fibroblast:melanoma co-cultures. Interaction between fibroblast-associated heparan sulphate and a heparin-binding protein on the surface of B16 melanoma cells leads to the release of a collagenase-stimulatory factor from the melanoma cells which then acts on the fibroblasts. Removal of the heparan sulphate proteoglycan from the surface of fibroblasts by heparanase treatment abrogated the effect.

Heparan sulphate proteoglycans have also been shown to be important in the mitogenic stimulation of Schwann cells by dorsal root ganglion neurons (Ratner et al., 1985) and haempoietic stem cells by bone marrow stromal cells (Roberts et al., 1988). Removal of the heparan sulphate by heparanase (in the case of the stromal cells) or

β-D-xyloside or heparanase (in the case of the neurons) inhibited the mitogenic effect.

Incidental to the possible role of the ECM in the melanoma cell:fibroblast interaction were the effects bFGF which, although not relevant to the main theme of this thesis, were nevertheless interesting, in that they have not been reported previously. Much work has been done on the proliferative capacity of bFGF but no one seems to have studied its effects on macromolecular synthesis. I have shown the bFGF consistently inhibited fibroblast collagen synthesis in dose-dependent manner, and stimulated fibroblast proliferation (Fig. 14).

Gospodarowicz, in discussing the biology of FGFs, has drawn attention to the fact that they decrease adhesion of normal fibroblasts to the substratum; they promote anchorage-independent growth of these cells; and they release the cells from contact inhibition to produce a "transformed" appearance (Gospodarowicz, 1987). A decrease in collagen synthesis (and as a result, a presumed decrease in fibronectin binding sites leading to diminished adhesion) and increased proliferation (with release from contact inhibition and "transformation") are both effects that one would intuitively associate with Gospodarowicz's account.

Heparin and bFGF are known to act synergistically in other systems (Konkle and Ginsburg, 1988; Ulrich et al., 1986); my results confirm this enhancing interaction (Table 13). It has been suggested that the

effect of heparin, in this particular context, is mediated by its capacity, shared with other proteoglycans, for stabilization and presentation of the growth factor (Saksela et al., 1988; Vlodavsky et al., 1987). I have no data to support or refute this reasonable hypothesis.

Of particular interest is the fact that the melanoma cells, while consistently stimulatory in the absence of FGF or heparin, synergized with the peptide to inhibit fibroblast collagen synthesis when the stimuli were delivered simultaneously. This synergy between melanoma cells and bFGF is readily explicable by postulating the deposition of melanoma cell matrix or the presence of matrix-like structures on the melanoma cell surface that are, like heparin, capable of presenting bFGF and so delivering a more effective signal. Such a mechanism would be entirely analogous to that suggested by the results of the edifying experiments done by Roberts et al. (1988) with haemopoietic stem cells and by Saksela et al. (1988) using bovine capillary endothelial cells.

APPENDIX

A.1 CELL CULTURE

A.1.1 Establishment of primary cultures

Tumours:

Primary cultures of tumours were established from samples removed at the time of surgery. Tumour pieces were placed into bottles containing serum-free RPMI and were immediately sent to the laboratory.

With sterile scissors, the specimen was minced finely and the tumour fragments transferred to a bottle containing 0.25% trypsin and 0.02% EDTA in TD (0.14M NaCl, 5mM KCl, 0.7mM Na_2HPO_4, 24.8mM Tris HCl pH 7.4). After 15 mins incubation at 37°C with gentle agitation, the supernatant was removed and added to an equal volume of RP-10 to neutralize the proteolytic activity of the trypsin. The cell suspension was centrifuged at 350 g for 5 min and the pellet resuspended in RP-10 and plated onto a tissue culture dish.

Any tumour tissue left undigested by the trypsin was incubated overnight at 4°C in a 3mg/ml solution of collagenase (Sigma C5138) in RP-5. The cell suspension was then centrifuged and plated onto a tissue culture dish as before.

After 24 hours the petri dishes were washed gently with fresh medium to remove non-adherent cells; these were pooled, centrifuged and reseeded in a new petri dish. Thereafter cultures were maintained and passaged on the basis of twice-weekly inspection under phase contrast microscopy. Standard criteria for passage and methods for tissue culture were employed (Freshney,1987).

Fibroblasts:

Foreskin fibroblast cultures were established from foreskins obtained from neonatal circumcision. The tissue was cut into 1 mm^2 fragments with a scalpel blade taking care not to compress the tissue or allow the fragments to become dry. Approximately 20-30 fragments were placed on a 60mm tissue culture petri dish in a small volume of medium such that the fragments were kept moist but remained attached to the dish. The next day, an additional 5ml of DB-10 was added. Epithelial cell outgrowth was observed after 1-5 days and this was followed by the appearance of fibroblasts. Once confluence had been reached (2-6 weeks), cells were passaged with 0.25% trypsin containing 0.02% EDTA and reseeded. Epithelial cells did not adhere following trypsinization and pure fibroblast cultures were obtained.

Rat embryo skin fibroblast cultures were established from 18-19 day old rat embryos. The skins were processed as for tumours.

A.1.2. Maintenance and storage of cell lines

Established cell lines were maintained in Dulbecco's modified Eagle's medium (DB; Gibco Cat. No. 074-2100) or RPMI-1640 (RP; Gibco Cat. No. 074-1800) supplemented with 10% FCS (heat-inactivated; 56°C for 30 min), 300μg penicillin/ml, 200μg streptomycin sulphate/ml and 10μg tylocine/ml (Gibco Cat. No. 043-05220H). The cultures were kept at 37°C in a humid atmosphere containing 5% CO_2 in air in a Hotpack CO_2 incubator (Hotpack Corporation, Philadelphia, PA, U.S.A.)

Cell lines cultured were examined twice weekly under phase contrast microscopy and passaged when they approached confluency. Passaging was performed by incubating the cells briefly (± 5 min) in 0.25% trypsin in TD at 37°C. The detached cells were dispersed by gentle pipetting and the suspension added to an equal volume of medium containing FCS to neutralize the protease. After centrifugation at 350 g for 5 min, the pellet was resuspended in medium and reseeded in new petri dishes.

For long-term preservation, cell stocks were frozen in liquid nitrogen by resuspending a cell pellet in medium containing 10% FCS and 10% DMSO. This suspension was distributed in 1ml volumes into screw-topped 38 x 12.5mm nylon tubes (Greiner 121261). The tubes were then frozen following the freezing protocol recommended by Farrant et al (1974).

A.1.3. Mycoplasma contamination

All the cell lines used were tested for mycoplasma contamination according to the method of Chen (1977). Cells were cultured for 7 days in medium from which tylocine had been omitted. Cells were then fixed with acetic acid:methanol (1:3) and stained with 0.5 μg/ml of bisbenzimide fluorochrome (Hoechst No. 33258) in Hank's balanced salt solution for 30 min. The cell layer was well rinsed with deionized water and mounted in buffer containing 0.02M citric acid, 0.06M disodium phosphate and 50% glycerol, pH 5.5. Cultures were examined with a Nikon fluorescence microscope.

A.1.4. Assessment of in vitro doubling time

Cells were seeded at low density (usually $1x10^5$ cells/35mm dish) in medium containing 10% FCS. Cell monolayers from duplicate dishes were trypsinized at 48hr intervals and the cells counted in a haemocytometer. Medium was replaced at 48hr intervals.

A.2. CELL LINES

A.2.1. Human malignant melanomas

UCT-Mel 1

The UCT-Mel 1 cell line was established from an inguinal metastasis of a 67 year old Caucasian female who presented with a primary malignant melanoma of Clark's level III on her right ankle. The

tissue sample for culture was a deeply pigmented lymph node and showed, on histological examination, the presence of epitheloid malignant melanoma with melanin being its prominent feature.

The cells in tissue culture were non-pigmented at low density and deeply pigmented at confluence. They grew with an in vitro doubling time of 41hrs and an in vivo doubling time of 6 days.

UCT-Mel 2

Tumour tissue was obtained from a metastatic axillary lesion, removed from a 67 year old Caucasian female. The sample for culture was non-pigmented and showed, on histological examination, an epitheloid type malignant melanoma with pigmentation visible in certain areas, while others were non-pigmented.

The cells were non-pigmented at low density, becoming deeply pigmented at confluence. They grew with an in vitro doubling time of 50hrs and an in vivo doubling time of 5 days.

UCT-Mel 3

This cell line was established from a metastatic liver deposit removed from a 71 year old Caucasian female. Histological examination of the tissue sample showed the presence of a metastatic malignant melanoma of epithelioid type with scanty intracellular melanin. The cells were non-pigmented at low density and at confluence. They grew with an in vitro doubling time of 58hrs and an in vivo doubling time of 8 days.

UCT-Mel 4

This cell line was established from a left inguinal lymph node removed from a 67 year old Caucasian female. The tissue sample was pigmented and showed, on histological examination, the presence of a deeply pigmented metastatic melanoma of epithelioid type. The cells in culture lost their ability to produce pigment, even at high density. They grew with an *in vitro* doubling time of 52hrs and an *in vivo* doubling time of 6 days.

UCT-Mel 5

Tumour tissue was obtained from a metastatic brain deposit removed from a 48 year old Caucasian female. The tissue sample was non-pigmented and showed, on histological examination, a metastatic malignant melanoma of a mixed spindle and epithelioid cell type.

The cells in culture were non-pigmented even at high density. They had an *in vitro* doubling time of 58hrs and an *in vivo* doubling time of 8-10 days.

UCT-Mel 7

The UCT-Mel 7 cell line was established from the femoral lymph node of a 52 year old African woman who presented with a chronic ulcer on the left heel. This was removed and diagnosed as malignant melanoma, Clark's level V. Histological examination of the sample for culture showed a non-pigmented secondary malignant melanoma with spindle cell morphology,minimal melanin production and the presence of extensive reticulin.

The cells in culture were non-pigmented, even at high density. Their in vitro doubling time varied with the number of passages, as did their in vivo doubling time.

RPMI 7272 (Bowes)

This melanoma cell line was originally established by Dr G. Moore of Denver, Colarado. A subculture of this line was provided by Dr E. Reich of the Rockefeller University, New York.

A.2.2 Human breast carcinomas

MDA-231

This cell line was established by Cailleau et al. (1974) from a pleural effusion obtained from a patient with adenocarcinoma of the breast.

T47-D

T47-D is a differentiated epithelial substrain of the the T47 line which was isolated by I. Keydar from a pleural effusion obtained from a patient with infiltrating ductal carcinoma of the breast.

MCF 7

This breast carcinoma cell line was isolated by Soule et al. (1973) from a pleural effusion obtained from a patient with breast adenocarcinoma.

ZR-75-1

This cell line was derived from a malignant ascitic effusion in a 63 year old, Caucasian female with infiltrating ductal carcinoma of the breast. (Engel et al. 1978)

A.2.3. Fibroblast cultures

Foreskin fibroblasts and rat embryo skin fibroblasts

These fibroblast cultures were established as primary cultures in this laboratory by the methods described in Section A.1.1.

Don

This diploid cell line was established from a lung biopsy from a normal male adult Chinese hamster (*Cricetulus griseus*). A subculture of this line was obtained from Dr A. Retief at Stellenbosch University Medical School, Cape Town.

3T3-J2

Mouse 3T3-J2 fibroblast culture was obtained from Dr H. Green

A.2.4. Macrophage cultures

$P388D_1$

$P388D_1$ is a mouse tumour line originally isolated by Dawe and Potter (1957) from a methylcholanthrene-induced lymphoid neoplasm (P388) in a DBA/2 mouse. It exhibits certain macrophage-like

characteristics such as phagocytosis; Fc and complement receptors; and cytotoxicity in an antibody-dependent, cell-mediated cytotoxic system. It lacks other attributes such as chemotactic responsiveness and the enzyme adenosine (Snyderman et al., 1977; Koren et al., 1975).

Peritoneal exudate cells

Resident mouse peritoneal exudate cells were collected from 6-8 week old female mice by lavage with 4-5ml of DB-1.

Elicited peritoneal exudate cells were harvested from animals in which a sterile peritonitis had been induced by intraperitoneal inoculation of:
Starch (2% suspension) or Latex (1:1000 dil) 5 days before harvesting;
Thioglycollate (3% broth) or LPS (60μg/ml) 4 days before harvesting;
FCS 1 day before harvesting.

A.3. NUDE MICE

A.3.1. Maintenance of athymic nude mice

Athymic nude mice of N:NIH (S) II - nu/nu strain (Azar et al., 1980) were bred in the UCT Animal Unit from breeding stocks imported in June 1980 and provided by Dr. B.C. Giovanella of the Cancer Research Laboratory, St. Joseph's Hospital, Houston, Texas, U.S.A. This strain has combined immunodeficiency i.e. in addition

to the thymic hypoplasia found in the original strain of nu/nu mice, both T-cell and B-cell zones of the lymph nodes and spleen are depleted of lymphocytes. The colony is maintained by breeding pairs of the heterozygous nu/+ females and homozygous nu/nu males. The homozygous female offspring were used for experiments.

The animals were housed in sterile plastic cages covered with non-woven spun polyester filter hoods (Laboratory Products Inc., Mainwood, New York) and provided with autoclaved food, water and bedding. The mice were fed a commercially prepared rodent food (mouse pellets, Epol (Pty) Ltd., Cape Town). The drinking water was supplemented with vitamins (0.5 ml/litre of Pancebrin, Lilly Pharmaceuticals) and antibiotics (27 mg/litre Cefamandole nafate, Lilly Pharmaceuticals). To avoid any possible effects of antibiotics on tumour growth, the Cefamandole was discontinued seven days before the start of any experiments.

The mice were handled under stringent sterile conditions at all times. All tumour cell inoculations and other manipulations were conducted in a laminar flow hood by personnel clothed in sterile gowns and wearing sterile rubber gloves. Athymic mice kept under these conditions thrived and had lifespans comparable to those of normal mice.

A.3.2 Inoculation into nude mice

Cells to be inoculated into the mice were released from the tissue culture dish with 0.25% trypsin containing 0.02% EDTA in a buffered salt solution. The trypsin was neutralized by the addition of foetal calf serum to a final concentration of 5%, and the cells were then pelleted, washed once with serum-free medium, and adjusted to give the requisite number of cells in an inoculum of 0.1 ml.

The cells were injected subcutaneously into the inter-scapular region of 6-8 week old mice. The animals were subsequently examined at weekly intervals when tumour growth was recorded. The tumour volume was calculated as the product of 3 major diameters.

When called for by the experimental protocol, tumours were removed under light ether anaesthesia and the skin incision closed with surgical clips that were removed after one week. Alternatively, the tumours were removed after killing the animals.

Each tumour was divided into several representative portions. One of these was placed in buffered formol saline for histology. The other fragments were variously used for re-inoculation, re-establishment in culture, biochemical analysis or cryopreservation (Farrant et al., 1974).

A.3.3. Analysis of tumours removed from nude mice

Histology of the Tumours

Formalin-fixed samples were embedded in paraffin, sectioned and stained according to conventional histological techniques (Mayers' haematoxylin and eosin). Sections of the tumours were stained for reticulin according to the method of Gordon and Sweets (1936).

Hydroxyproline content of the tumours

The hydroxyproline content of the tumours was determined using the method of Hutterer and Singer (1960) in which 10 mg of tumour tissue was hydrolysed in 1 ml 6N HCl at 121°C for 16 hrs. The hydrolysates were evaporated to dryness under vacuum and the residue dissolved in 1 ml water. Samples so obtained were assayed for hydroxyproline content by adding p-dimethylaminobenzaldehyde, reading the absorbance at 500nm and 560nm and referring to a standard curve.

Phosphoglucoisomerase analysis

Lysates were prepared by homogenizing the tumours in 2 volumes of 0.1M sodium phosphate pH 7.4. Samples were loaded onto 11% starch gels, using inserts of Whatman's No. 3 chromatography paper, and electrophoresed at 8 V/cm for 5 hrs, with cooling. The buffer system used was as follows:

0.2M Tris, 0.053M citric acid pH 6.5 for the tray buffer. Gel buffer was a 1:15 dilution of tray buffer. Phosphoglucoisomerase (PGI) isoenzymes moved towards the cathode.

After electrophoresis the gel was stained with the following reaction mixture:

0.2M Tris HCl pH 8.0	10ml
Fructose-6-phosphate	10mg
NADP	5mg
Glucose-6-phosphate dehydrogenase	1.4U in 10μl
Methyl Thiazolyl tetrazolium (MTT)	5mg in 1ml H_2O
Phenazine methosulphate	5mg in 0.5ml H_2O
1.8% Agar	10ml

The PGI enzyme converts fructose-6-phosphate to glucose-6-phosphate which is then dehydrogenated with the transfer of hydrogen to NADP. The NADP is regenerated by the transfer of hydrogen to MTT to give the insoluble blue dye, formazan.

A.4. COLLAGEN ASSAY

Incorporation of [^{3}H]-Proline into collagen was assayed by the method of Webster and Harvey (1979).

Foreskin fibroblasts were plated at 1x10^5/35mm tissue culture dish (Falcon 3001) in DB-10 and used when confluent (usually 2-3 days

after plating). Confluent fibroblast monolayers were cultured either alone or as co-cultures for 24 hours in 1ml RP-10. Co-cultures were established by plating either UCT-Mel 7 cells ($3x10^5$/35mm dish) or peritoneal macrophages ($1x10^6$/35mm dish) on top of confluent fibroblast monolayers at time 0. The medium was then changed to 1ml serum-free RPMI, supplemented with 25μg/ml ascorbate (Merck 127) and, in some cases, 220μg/ml proline (Merck 7434). The cultures were then preincubated for 4 hours and then labelled for 16 hours with 5-10μCi [^{3}H]-Proline (15-40 Ci/mmol; Amersham TRK 323). The medium was collected and assayed for radioactive collagen.

To 1ml of radiolabelled medium, I added 100μl 5M acetic acid and 100μl pepsin (5mg/ml in 0.5M acetic acid; Sigma P-7012) and then incubated the samples at 4°C overnight with shaking. All subsequent steps were performed at 4°C. The next day, I added 1ml neutral salt-soluble rat skin collagen (0.5mg/ml in 0.5M acetic acid) as carrier and made the volume up to 3 ml with 0.5M acetic acid. The collagen was precipitated by the addition of 750μl 25% NaCl in 0.5M acetic acid. After incubating for 2 hours, the samples were centrifuged at 3000g for 30 mins. The pellet was re-dissolved in 1ml 0.15M NaCl in 0.05M Tris HCl pH 7.5 and the collagen re-precipitated by the addition of 10ml 4.5M NaCl in 0.05M Tris Hcl pH 7.5. The samples were incubated and centrifuged as before. The pellet was washed in 10ml 20% ethanol. The final precipitate was dissolved in 1ml 0.5M acetic acid and counted in a liquid scintillation counter.

A.4.1. Preparation of neutral salt-soluble carrier collagen

Rat skin collagen was purified by the method of Gross (1958). Rats were killed by CO_2 inhalation. The skins were shaved, removed from the animal and cleared of subcutaneous fat and tissue by scraping with a scalpel blade. All subsequent steps were performed at 4°C.

The skins were minced in a meat grinder and extracted by shaking for 16 hours in 2 volumes of 0.45M NaCl. The suspension was centrifuged at 40 000g for 1 hour, and the supernatant filtered through several layers of surgical gauze and then through a porosity 2, pyrex sintered glass filter. The filtrate was dialysed against four changes of 0.45M NaCl over 2 days.

The concentration of NaCl in the solution was brought to 16% by the slow addition, with stirring, of solid salt and the solution was left to stand for 6 hours. The precipitate was pelleted by centrifugation (7000g; 30 min) and dissolved in distilled water such that the concentration of NaCl was approximately 0.4M. The resulting turbid solution was dialysed against 0.067M sodium phosphate pH 7.6 for 12 hours with 3 changes. The small amount of insoluble material that formed after dialysis was removed by centrifugation at 1000g for 30 min. The collagen was reprecipitated in this manner three times. The final precipitate was dialysed against 0.5 M acetic acid and adjusted to give a final concentration of 0.5mg/ml.

A.5. SUPEROXIDE ANION PRODUCTION

Superoxide anion secretion was assayed by the superoxide dismutase-inhibitable reduction of ferricytochrome c. (Johnston et al., 1978). UCT-Mel 7 cells and foreskin fibroblasts were plated at $1x10^5$ cells/16mm well in 1ml Hanks balanced salt solution without phenol red (Gibco 076-1201) and supplemented with 5.5mM glucose, (BDH 10117), 25mM Hepes (Boehringer Mannheim 223778), MEM amino acid mix (50x conc.; Gibco 043-1130H), L-glutamine (Sigma G3126) and MEM vitamin mix (100x conc.; Gibco 043-1120H). Thioglycollate-elicited mouse peritoneal macrophages were harvested by peritoneal lavage of BALB/c mice and plated at $8x10^5$ cells/16mm well. Non-adherent cells were removed by washing after 2 hours incubation.

At the start of the experiment ferricytochrome c (Sigma C-2506) was added to give a final concentration of 10^{-5} M. To some assay wells was added superoxide dismutase (Oxinorm) at a final concentration of 150U/ml and to other wells tetradecanoyl phorbol acetate (PMA; Consolidated Midland Corporation) at a final concentration of 10ng/ml.

The cells were incubated at 37°C in 5% CO^2 - 95% air. At the indicated times, the reaction mixture was removed from the wells, centrifuged at 3000g in an Eppendorf microcentrifuge and the optical density measured at 550nm. The concentration of cytochrome c reduced was determined using the equation $\Delta E_{550nm} = 21\ mM^{-1}\ cm^{-1}$.

At the end of the experiment, the cells were digested with 1M NaOH and 10% Na deoxycholate (O/N at 37°C) and the protein content of the digest was determined by the method of Lowry et al. (1957) using BSA as standard.

A.6 PLASMINOGEN ACTIVATOR ASSAY

Plasminogen activator was assayed as described by Wilson and Dowdle (1978). The assay relies on the measurement of plasminogen dependent release of soluble, radioactive fibrin degradation peptides from an insoluble substrate of [^{125}I]-labelled fibrin adhering to the surface of a plastic well.

[^{125}I]-labelled human fibrinogen (30μg fibrinogen/well; 120-130 000 cpm/well) was used to coat the surface of the wells and the plates (24-well multiwell plates) were dried at 37°C for 3 days before use.

[^{125}I]-fibrinogen was converted to fibrin by the addition of 1ml Eagle's Minimal essential medium (Gibco Cat. No. 072-1100). After incubation at 37°C for 2 hours, the wells were washed twice with PBS and once with 0.1M Tris HCl pH 8.1.

For the assay each well contained 2μg of purified human plasminogen and 80μg of BSA in a final volume of 300μl of 0.1M Tris HCl pH 8.1. Twenty microlitre aliquots of harvest fluid were added to initiate the reaction.

Control wells contained 1) 300µl trypsin (to give the total radioactivity present in each well); 2) 20µl of sample without plasminogen (to detect plasminogen-independent proteolysis) and 3) plasminogen without sample (to detect background lysis due to plasmin contamination of the plasminogen solution). Standard wells included in each assay contained urokinase in 12 doubling dilutions, usually commencing with 0.2 Ploug units per well.

The multi-well plates were incubated at 37°C in a humid atmosphere. Fibrinolysis was monitored by measuring the solubilized radioactivity in 50µl aliquots at three separate time points.

For each time point, the urokinase standard curve was plotted and sample values falling on the linear range of the standard curve were determined and expressed as UK units per assay well.

A.7. MEASUREMENT OF MACROMOLECULAR SYNTHESIS

After a 5 hour pulse with [^{3}H]-Leucine (5µci/ml; Amersham TRK 170), [^{3}H]-Uridine (5µCi/ml; 1µg/ml; Amersham TRK 410) or [^{3}H]-Thymidine (5µCi/ml; 1µg/ml; Amersham TRK 61) the cultures were washed 3 times with 2ml PBS and the cells released by incubation in 1ml of 0.25% trypsin. An aliquot (10µl) of the suspension was taken for cell counting; the culture dishes were placed on ice; and 1ml of ice-cold 10% TCA was added. The dishes were kept at 4°C for 30 min. The TCA precipitate was collected on a Whatman GF/c filter and washed with 30ml of ice-cold 5% TCA. The filter was dried overnight in a hot oven and radioactivity measured, following the

addition of 5ml of Instagel, in a Packard liquid scintillation counter.

A.8. MONOCLONAL ANTIBODY PRODUCTION

Monoclonal antibodies were raised against UCT-Mel 7 cells and foreskin fibroblasts. BALB/c mice were immunized by intraperitoneal injection of $4x10^6$ UCT-Mel 7 cells or $3x10^6$ fibroblasts in complete Freund's adjuvant. Two weeks later they received a second similar injection in incomplete Freund's adjuvant. A booster injection of cells in PBS was given 3-4 days before the fusion.

The immune spleens were removed aseptically and single cell suspensions of the splenocytes were prepared and then fused with SP2 myeloma cells using standard techniques (Campbell, 1984). Clones were screened in an ELISA assay against 1-5 x 10^4 UCT-Mel 7 cells or fibroblasts attached to a microtitre well with poly-L-lysine and fixed with 0.1% glutaraldehyde.

The final clone, which was selected and used to make ascitic fluid, was specific for the cell type against which it was raised. The monoclonal antibody, αFIB-D7, recognized fibroblasts and not UCT-Mel 7 cells. The monoclonal antibody, αNM-B12, was specific for UCT-Mel 7 cells and did not recognize fibroblasts.

A.9. CONJUGATION OF ANTIBODY TO ERYTHROCYTES

Monoclonal antibodies were conjugated to erythrocytes according to the method of Parish and McKenzie (1978). Human or sheep erythrocytes were washed 4 times in saline by repeated centrifugation and resuspension. Washed packed red cells (250μl) were added to a tube containing 1.6mg of the appropriate antibody in 4ml of saline. Chromic chloride (BDH 10078; 0.4ml of a 0.1% solution in 0.9% NaCl) was then added and the coupling reaction allowed to proceed at room temperature for 5min. The reaction was stopped by the addition of 7ml of PBS. The conjugated erythrocytes were washed once in PBS and resuspended in 12.5ml RP-10. They could be kept at 4°C for up to 24 hours without loss of effectiveness.

A.10. SEPARATION OF A MIXED CELL POPULATION USING CONJUGATED ERYTHROCYTES

After the 24 hours co-culture, the cells were released with trypsin. The trypsinized cells were resuspended in RPMI-10, mixed with an equal volume of conjugated red blood cells and incubated at room temperature for 15 minutes. The cell mixture was layered onto a Ficoll-Hypaque cushion and spun at 1000g for 15 minutes. The pellet contained the conjugated red blood cells and the cells recognized by the antibody conjugated to erythrocytes. The interface contained the cells not recognized by the antibody. The cells from the interface could be harvested and used in further

experiments. A rapid and efficient separation of the 2 cell types was thus achieved.

A.11. EXTRACTION OF RNA AND NORTHERN BLOT ANALYSIS

Total RNA was extracted using guanidinium thiocyanate (Fluka 50990) as described by Chirgwin et al. (1979). The quality and quantity of the extracted RNA was checked by spectrophotometric analysis at 260nm and 280nm.

Twenty micrograms of RNA were heat denatured in formamide (Merck 9684), electrophoresed in a 1.5% agarose gel containing 2.8M formaldehyde (BDH 10113) and transblotted electrophoretically onto nitrocellulose paper (Hybond N, Amersham RPN 303N) at 300mA for 16 hours in 25mM sodium phosphate buffer pH 6.5 (Amersham booklet: Membrane transfer and detection methods). The RNA was fixed to the Hybond by a 5 min exposure to UV light on a standard UV transilluminator.

The recombinant plasmids, Hf 677 (Chu et al. 1982) and Hf 32 (Myers et al. 1981), containing cDNA sequences specific for the human α1(I) and α2(I) collagen genes, respectively, were the gift of Dr. F. Ramirez, Rutgers Medical School, Piscataway, N.J. The recombinant plasmid, pHF β A-1 (Gunning et al., 1983), containing cDNA sequences specific for human cytoplasmic β-actin, was the gift of Dr I. Parker, University of Cape Town, Medical School. The cDNA plasmid probes were nick-translated with [α-^{32}P]dCTP (Amersham PB 10205) using the Amersham nick translation kit (Amersham N 5000). The

nitrocellulose filters were incubated in prehybridization buffer for 6 h at 55°C. The nick-translated probes were denatured at 100°C for 5 min, cooled, and added to the prehybridization solution. Following hybridization for 16 h at 55°C, the RNA blots were washed extensively with washes of increasing stringency. The final wash was in 0.5X SSPE containing 0.1% SDS at 68°C for 15 min. The blots were then wrapped in Saran wrap and put up for autoradiography at -80°C using X-ray film.

TABLE A.1 ANALYSIS OF VARIANCE: DEXAMETHASONE AT 10^{-6}M

ADDITIONS	SUM SQUARES	DEG FREEDOM	MEAN SQUARES	F-TEST RATIO	P
Expt. No. 1:					
UCT-Mel 7	3.093718	1	3.093718	121.3515	<0.001
Dex	.1384564	1	.1384564	5.430969	0.05
Dex x Mel 7	.2293625	1	.2293625	8.996774	0.025
Error	.2039509	8	2.549386E-02		0.025
Expt. No. 2:					
UCT-Mel 7	.1458597	1	.1458597	22.41158	<0.001
Dex	.8263512	1	.8263512	126.9702	<0.001
Dex x Mel 7	2.349758E-02	1	2.349758E-02	3.610441	NS
Error	5.206585E-02	8	6.508231E-03		
Expt. No. 3:					
UCT-Mel 7	1.083002	1	1.083002	67.54256	<0.001
Dex	.3376808	1	.3376808	21.05982	<0.005
Dex x Mel 7	9.19342E-04	8	9.19342E-02	5.733573E-02	NS
Error	.1282749		1.603437E-02		

TABLE A.2 ANALYSIS OF VARIANCE: DEXAMETHASONE AT 10^{-7}M

ADDITIONS	SUM SQUARES	DEG FREEDOM	MEAN SQUARES	F-TEST RATIO	P
Expt. No. 1:					
UCT-Mel 7	1.390602	1	1.390602	12.88015	<0.01
Dex	.5542703	1	.5542703	5.133806	NS
Dex x Mel 7	7.410383E-02	1	7.410383E-02	.6863705	NS
Error	.8637182	8	.1079648		
Expt. No. 2:					
UCT-Mel 7	.2514286	1	.2514286	42.2554	<0.001
Dex	4.775238E-02	1	4.775238E-02	8.025324	0.025
Dex x Mel 7	1.144409E-03	1	1.144409E-03	.1923308	NS
Error	.0476017	8	5.950213E-03		
Expt. No. 3:					
UCT-Mel 7	1.382444	1	1.382444	55.65477	<0.001
Dex	4.876709E-02	1	4.876709E-02	1.963277	NS
Dex x Mel 7	1.098061E-02	1	1.098061E-02	.4420598	NS
Error	.1987171	8	2.483964E-02		

TABLE A.3 ANALYSIS OF VARIANCE: DEXAMETHASONE AT 10^{-8}M

ADDITIONS	SUM SQUARES	DEG FREEDOM	MEAN SQUARES	F-TEST RATIO	P
Expt. No. 1:					
UCT-Mel 7	3.050211	1	3.050211	89.88564	<0.001
Dex	.2374497	1	.2374497	6.997323	0.05
Dex x Mel 7	.217617	1	.217617	6.412883	0.05
Error	.2714749	8	3.393436E-02		
Expt. No. 2:					
UCT-Mel 7	.5030671	1	.5030671	37.7186	<0.001
Dex	.5088177	1	.5088177	38.14977	<0.001
Dex x Mel 7	3.030396E-02	1	3.030396E-02	2.272108	NS
Error	.106699	8	1.333737E-02		
Expt. No. 3:					
UCT-Mel 7	1.281839	1	1.281839	140.0585	<0.001
Dex	6.541443E-02	1	6.541443E-02	7.14742	0.05
Dex x Mel 7	3.746033E-03	1	3.746033E-03	.4093053	NS
Error	.0732174	8	9.152174E-03		

TABLE A.4 ANALYSIS OF VARIANCE: RETINOIC ACID

ADDITIONS	SUM SQUARES	DEG FREEDOM	MEAN SQUARES	F-TEST RATIO	P
10^{-6}M:					
UCT-Mel 7	2.285515	1	2.285515	33.34508	<0.001
R.A.	.2543335	1	.2543335	3.710661	NS
R.A. x Mel 7	.2714996	1	.2714996	3.961111	NS
Error	.5483303	8	6.854129E-02		
10^{-7}M:					
UCT-Mel 7	2.392347	1	2.392347	34.12644	<0.001
R.A.	.6320438	1	.6320438	9.016002	0.025
R.A. x Mel 7	.3091221	1	.3091221	4.409576	NS
Error	.5608196	8	7.010246E-02		
10^{-8}M:					
UCT-Mel 7	2.060892	1	2.060892	27.20886	<0.001
R.A.	1.001673	1	1.001673	13.22455	0.01
R.A. x Mel 7	.1978893	1	.1978893	2.612627	NS
Error	.6059475	8	7.574344E-02		

TABLE A.5 ANALYSIS OF VARIANCE: INDOMETHACIN

ADDITIONS	SUM SQUARES	DEG FREEDOM	MEAN SQUARES	F-TEST RATIO	P
10^{-6}M:					
UCT-Mel 7	1.878624	1	1.878624	32.84406	<0.001
Indo	2.464295E-03	1	2.464295E-03	4.308336E-02	NS
Indo x Mel 7	2.029419E-03	1	2.029419E-03	3.548041E-02	NS
Error	.4575863	8	5.719829E-02		
10^{-7}M:					
UCT-Mel 7	2.126894	1	2.126894	273.7714	<0.001
Indo	4.108429E-03	1	4.108429E-03	.5288323	NS
Indo x Mel 7	1.763153E-02	1	1.763153E-02	2.269511	NS
Error	6.215096E-02	8	7.76887E-03		
10^{-8}M:					
UCT-Mel 7	3.46258	1	3.46258	182.1834	<0.001
Indo	4.037285E-02	1	4.037285E-02	2.124215	NS
Indo x Mel 7	.2864399	1	.2864399	15.07101	0.005
Error	.1520481	8	1.900601E-02		

TABLE A.6 ANALYSIS OF VARIANCE: PMA AT 10ng/ml

ADDITIONS	SUM SQUARES	DEG FREEDOM	MEAN SQUARES	F-TEST RATIO	P
Expt. No. 1:					
UCT-Mel 7	.5465622	1	.5465622	36.03855	<0.001
PMA	3.422943	1	3.422943	225.6978	<0.001
PMA x Mel 7	.1287499	1	.1287499	8.489349	0.025
Error	.1213284	8	1.516604E-02		
Expt. No. 2:					
UCT-Mel 7	.2439604	1	.2439604	26.70226	<0.001
PMA	1.193852	1	1.193852	130.671	<0.001
PMA x Mel 7	3.887463E-02	1	3.887463E-02	4.254955	NS
Error	7.309056E-02	8	9.136319E-03		

TABLE A.7 ANALYSIS OF VARIANCE: PMA AT 1ng/ml

ADDITIONS	SUM SQUARES	DEG FREEDOM	MEAN SQUARES	F-TEST RATIO	P
Expt. No. 1:					
UCT-Mel 7	1.027844	1	1.027844	59.54054	<0.001
PMA	1.242921	1	1.24921	71.99939	<0.001
PMA x Mel 7	7.106781E-03	1	7.106781E-03	.4116786	NS
Error	.1381035	8	1.726294E-02		
Expt. No. 2:					
UCT-Mel 7	.1363216	1	.1363216	10.60456	<0.025
PMA	.3913245	1	.3913245	30.44144	<0.001
PMA x Mel 7	5.249501E-03	1	5.249501E-03	.4083628	NS
Error	.1028399	8	1.285499E-02		

TABLE A.8 ANALYSIS OF VARIANCE: PMA AT 0.1ng/ml

ADDITIONS	SUM SQUARES	DEG FREEDOM	MEAN SQUARES	F-TEST RATIO	P
Expt. No. 1:					
UCT-Mel 7	1.752611	1	1.752611	81.23621	<0.001
PMA	2.184296E-02	1	2.184296E-02	1.012455	NS
PMA x Mel 7	5.096436E-02	1	5.096436E-02	2.362276	NS
Error	.1725941	8	2.157426E-02		
Expt. No. 2:					
UCT-Mel 7	.1287546	1	.1287546	8.929167	<0.025
PMA	6.795216E-02	1	6.795216E-02	4.7125	NS
PMA x Mel 7	3.851891E-03	1	3.851891E-03	.2671296	NS
Error	.1153565	8	1.441956E-02		

TABLE A.9 - EFFECT OF bFGF AND HEPARIN

ADDITIONS	COLLAGEN SYNTHESIS (μmoles proline/10^8 cells/16 hr) FIBROBLASTS ALONE	FIBROBLASTS CO-CULTURED
Expt. No. 1:		
None	0.90	1.41
bFGF (40ng/ml)	0.17	0.27
Heparin (20μg/ml)	0.37	0.54
bFGF + heparin	0.14	0.18
Expt. No. 2:		
None	0.562	1.168
bFGF (2.5ng/ml)	0.559	0.926
Heparin (10μg/ml)	0.498	0.708
bFGF + heparin	0.257	0.303

TABLE A.10 ANALYSIS OF VARIANCE: UCT-Mel 7, HEPARIN AND bFGF

ADDITIONS	SUM SQUARES	DEG FREEDOM	MEAN SQUARES	F-TEST RATIO	P
Expt. No. 1:					
UCT-Mel 7	.2496963	1	.249693	50.92891	<0.001
Heparin	.8656406	1	.8656406	176.559	<0.001
bFGF	2.259521	1	2.259521	460.8597	<0.001
Mel 7 x Hep	.0630374	1	.0630374	12.85732	0.01
Mel 7 x bFGF	.1137123	1	.1137123	23.19315	<0.001
Hep x bFGF	.6188879	1	.61888879	126.2305	<0.001
Mel 7 x Hep x bFGF	3.270865E-02	1	3.270865E-02	6.671368	0.05
Error	7.844544E-02	16	4.90284E-03		
Expt. No. 2:					
UCT-Mel 7	.5707264	1	.5707264	84.82561	<0.001
Heparin	.7938853	1	.7938853	117.9931	<0.001
bFGF	.3017292	1	.3017292	44.84525	<0.001
Mel 7 x Hep	.1892128	1	.1892128	28.12222	<0.001
Mel 7 x bFGF	5.930043E-02	1	5.930043E-02	8.813671	0.025
Hep x bFGF	6.232167E-02	1	6.232167E-02	9.262711	0.025
Mel 7 x Hep x bFGF	2.463341E-03	1	2.463341E-03	.3661201	NS
Error	.1076517	16	6.728232E-03		

TABLE A.11 ANALYSIS OF VARIANCE: EFFECT OF PROTOCOL

ADDITIONS	SUM SQUARES	DEG FREEDOM	MEAN SQUARES	F-TEST RATIO	P
Expt. No. 1:					
UCT-Mel 7	.7085881	1	.7085881	66.70709	<0.001
bFGF	4.489661E-02	1	4.489661E-02	4.226604	NS
Mel 7 x bFGF	4.296065E-02	1	4.296065E-02	4.044351	NS
Error	8.497906E-02	8	1.062238E-02		
Expt. No. 2:					
UCT-Mel 7	2.539206	1	2.539206	135.1103	<0.001
bFGF	3.046179	1	3.046179	162.0861	<0.001
Mel-7 x bFGF	9.183884E-03	1	9.183884E-03	.4886713	NS
Error	.1503487	8	1.879358E-02		

TABLE A.12 ANALYSIS OF VARIANCE: EFFECT OF β-D-XYLOSIDE

ADDITIONS	SUM SQUARES	DEG FREEDOM	MEAN SQUARES	F-TEST RATIO	P
Expt. No. 1:					
UCT-Mel 7	.1654389	1	.1654389	49.01816	<0.001
Xylo	.4036989	1	.4036989	119.6126	<0.001
Xylo x Mel 7	2.755356E-02	1	2.755356E-02	8.163888	0.025
Error	2.700043E-02	8	3.375054E-03		
Expt. No. 2:					
UCT-Mel 7	2.179419	1	2.179419	105.3637	<0.001
Xylo	2.650799	1	2.650799	128.1525	<0.001
Xylo x Mel 7	4.577637E-04	1	4.577637E-04	2.213053E-02	NS
Error	.1654778	8	2.068472E-02		

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